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Mutagenicity and Cytotoxicity of Irradiated Foods and Food Components*

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The preservation of foods by treatment with ionizing radiation can significantly increase the world's food resources by reducing spoilage and waste. However, irradiation can bring about chemical transformations in food and food components resulting in the formation of potential mutagens, particularly hydrogen peroxide and various organic peroxides. In order to evaluate the safety of irradiated foods for general consumption by the public, and, indeed, the safety of all foods subjected to environmental factors such as food additives, pesticides, drugs, air and water pollutants, etc., it is necessary to supplement the usual feeding tests with procedures designed to detect all classes of genetic damage. This article includes a comprehensive critical review of (1) the experimental evidence relating to the presence of mutagenic and cytotoxic agents in irradiated media, as detected by their effects on mammalian and non-mammalian cells; (2) the chemical changes produced in irradiated media, especially those which produce known mutagenic substances; and (3) new and convenient in vivo *methods for the detection and evaluation of genetic damage in mammals.*

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INTRODUCTION

The preservation of foods by ionizing radiation is of universal interest because it can significantly increase the world's food resources by reducing spoilage and waste (Joint FAO/IAEA/WHO Expert Committee on the Technical Basis for Legislation on Irradiated Food, 1966; International Symposium on Food Irradiation, 1966; Kraybill & Whitehair, 1967; Metlitskii, Rogachev & Krushchev, 1967). Another potential advantage of food irradiation is the possibility that, in some cases, the use of chemicals, especially the persistent pesticides derived from chlorinated hydrocarbons, would diminish. For example, the use of lindane for controlling insect infestation in wheat grain could be eliminated completely by applying low-dosage irradiation treatment.

The large-scale or commercial use of food-irradiation processes requires adequate scientific and technical data to permit the evaluation of the safety of irradiated foods to the consumer. In the past, tests of toxicity in all kinds of environmental factors—food additives, pesticides, drugs, polluted air and water—have measured acute, short-term, and chronic toxicity in terms of growth rate, reproductive capacity, pathological changes, blood counts, etc. However, it is now realized, especially since the thalidomide

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episode, that such protocols do not detect the more subtle population hazards such as mutagens and teratogens. Many substances to which the population has been exposed have been classified as safe only because the testing protocol omitted tests for assessing such damage.

When the Joint FAO/IAEA/WHO Expert Committee . . . (1966) considered reports of an increase in mutation rate in *Drosophila melanogaster* grown on irradiated media, they concluded that " the evidence at present available is insufficient to establish whether substances present in irradiated food may be mutagenic in man and that, at present, no significant hazard can be foreseen in relation to the safety for consumption of food irradiated in accordance with approved practice. However, more data concerning a possible mutagenic effect are desirable and if further research should substantiate the evidence now available, especially if such experimental work should suggest that the effect may also occur in mammalian species, the subject will need further consideration."

Since those words were written, several significant developments have taken place. These have included: (1) the availability of several practical mutagenic tests which can be performed *in vivo* in mammals, thus taking into account the metabolic transformations undergone by a compound after being ingested; (2) new experiments which prove unambiguously that mutagenic and cytotoxic substances are produced in many foods, food components and natural products subjected to irradiation; (3) identification of some of the chemical compounds responsible for these mutagenic and cytotoxic manifestations; and (4) an increasing recognition of the consequences resulting from the exposure of all segments of the human population to highly mutagenic substances (Crow, 1968).

The application of mutagenic tests is not limited to irradiated foods (Joint FAO/IAEA/WHO Expert Committee . . . , 1966) but is under consideration for all substances and all processes, such as heating, used in the treatment of food. None of the available *in vivo* mutagenic tests, to be described later, is, individually, completely satisfactory for the interpretation of effects and their extrapolation to man. However, when carefully done, such tests collectively reduce the possibility that important classes of genetic damage will escape detection.

A variety of cells, mammalian, plant and bacterial, may be killed or injured when they are placed in media previously exposed to ultraviolet or ionizing radiations (Hollaender, 1954, 1955; Stone, 1955). Such profound alterations in molecules and organisms produced indirectly by the prior irradiation of their environment have been called a " medium effect ", an " indirect effect " or an " after-effect ". Generally, however, the influence of direct irradiation on an organism or cell is far greater than that of indirect irradiation. Most investigations of medium effects using food or food components have been carried out *in vitro* and numerous mutagenic and cytotoxic responses [1] have been observed. While *in vitro* studies may demonstrate that harmful substances are produced by irradiation, this does not necessarily mean that those substances will be harmful *in vivo*. There are several possible reasons for this, including metabolic transformation. Even if a presumptive mutagenic agent is produced, it may not reach the target organs or cells in concentrations high enough to produce genetic damage. Relatively few *in vivo* studies on mammals have been performed in which the mutagenic manifestations, e.g., chromosomal aberrations or dominant lethal mutations, have been examined.

In view of the serious consequences to the human population which could arise from a high level of induced mutations it is desirable that protocols for irradiated foods should include *in vivo* tests on mammals for possible mutagenicity (for background information, references to the literature and the evaluation of human risks see United Nations (1962, 1966); Bartalos (1968) and Goldstein, Aranow & Kalman (1968)). Of the several tests now available, 3 seem to be particularly appropriate, even though modifications and further developments are still taking place. They are as follows: (1) the dominant lethal mutation test (Bateman, 1958, 1966; Epstein & Shafner, 1968; Generoso & Russell, 1969) involving the sequential mating of male animals fed irradiated food with untreated virgin females, which are subsequently dissected at mid-term of pregnancy; (2) the host-mediated assay (Gabridge & Legator, 1969; Gabridge, Denunzio & Legator, 1969a, 1969b) in which bacteria, *Neurospora*, or other organisms, implanted in the peritoneal cavity of a mammal fed

[1] Mutagenic response is defined here as " induced changes in the hereditary material of germ or somatic cells ". These changes can be classified as microlesions in which the changes, e.g., point mutations, are not visible under the light-microscope and involve changes within a gene, and macrolesions, in which the changes, e.g., chromosomal aberrations which involve several genes, can be seen under the light-microscope. Cytotoxic response is defined as cell damage, regardless of the mechanism, which results in inhibition of cell growth or in cell death.

with the irradiated food, are subsequently removed and examined for mutations; (3) bone marrow and testicular examination of chromosomes by cytogenetic techniques (Moorehead & Nowell, 1964; Pan & Wald, 1963; Pan, personal communication) following the feeding of the irradiated substance to test animals. General procedures and protocols for performing these tests for mutagenicity are described in the Annex.

In this article, the results and interpretation of experiments dealing with the mutagenic and cytotoxic manifestations of irradiated food and food components are reviewed. A description of the principles and application of *in vivo* mutagenicity testing is also included, together with brief descriptions of radiation chemical reactions occurring in irradiated media, particular emphasis being given to the nature of the presumptive mutagenic and cytotoxic agents. In some cases, mutagenic or cytotoxic agents have been positively identified and isolated. Accordingly, in view of the new information now available, it is possible to evaluate the public health safety of irradiated foods more accurately. It is hoped that the information assembled in this review, for the first time, will facilitate such studies.

MEDIUM EFFECTS

Food is a complex mixture of substances of low and high molecular weights, and when it is subjected to ionizing radiation the resulting chemical changes reflect the responses of the individual molecules as well as the interactions between the radiolytic degradation products and newly formed, or modified, products. The literature contains numerous reports of studies in which biologically deleterious effects were noted when foods, nutrient media, aqueous solutions, or even water itself, were subjected to ultraviolet or ionizing radiation and subsequently placed in contact with various organisms or macromolecules. In many cases, these medium effects were attributed to the formation of hydrogen peroxide, hydroperoxides, dialkyl peroxides, to a combination of these peroxides, or to compounds such as formic acid, formaldehyde or glyoxal.

Medium effects are manifested in many ways, such as modification of macromolecules, genetic effects (mutations and chromosome deletions), inhibition of bacterial and cell growth and the modification of a variety of biochemical and physiological processes. Scientific literature dealing with these effects and listing key references includes proceedings of symposia on implications of organic peroxides in radiobiology (Latarjet, 1958; Feinstein, 1963), a review by Scarascia-Mugnozza, Natarajan & Ehrenberg (1965) on the genetic effects produced by irradiated food and food components, a review article by Stone (1955) on the genetic effects of irradiated media, the development of bacteria and viruses (Latarjet, 1956), and an early review by Arnow (1936) on proteins and amino acids. In the past few years, several important articles dealing with the influence of irradiated media on cell growth and the induction of chromosome aberrations by irradiated media have been published; these are discussed later.

Extreme care must be exercised in interpreting the mechanism of effects produced by irradiated media. For example, a non-irradiated medium with the addition of a single long-lived radiolytic product such as hydrogen peroxide may, under certain conditions, mimic an irradiated medium to a large extent, but even here the agreement will usually be qualitative inasmuch as the irradiation of aqueous systems produces many other radiolytic products capable of affecting the test organism. Obviously, the nature of the medium also depends upon the kind of effect measured and the specific characteristics of the test organism itself.

The addition of hydrogen peroxide to a non-irradiated medium is an inadequate test with which to evaluate whether radiolytic hydrogen peroxide contributes to, for example, the cytotoxicity of irradiated sucrose solutions. One basis for the planning of suitable controls is the fact that hydrogen peroxide may react more readily with molecules produced by radiolysis than with the parent molecules.

There are many other factors which can, and do, modify the biological effects of irradiated media. For example, the pH of many media decreases after irradiation and may, therefore, stabilize a toxic product and, depending on the pH, some toxic agents may be hydrolysed and destroyed during storage. The factors which must be considered in the design and interpretation of experiments on medium effects have been reviewed by Schubert (1967).

The comparison of the work on medium effects of different investigators is beset by many pitfalls. Consider the cytotoxic effects of irradiated media on the growth of bacteria; using the same system, 2 reliable investigators may obtain opposite results. Often, the reason lies in the cell concentrations employed; at high cell concentrations, no anti-

bacterial action of an irradiated medium incorporated into the growth medium may be observed. This apparently is due to a detoxification action by the cells themselves. Similar considerations apply to cytotoxic and mutagenic studies *in vitro* using cells of different origin, including mammalian cells. Obviously, it is worth while running all cytotoxicity and mutagenicity tests over a range of cell concentrations.

Studies on medium effects should include a measure of the average concentration of energy expended per cell. For example, in the case of a known cytotoxic or mutagenic agent, it is advisable to record the micromoles of the chemical per cell. In the case of ionizing radiation, the number of units of absorbed energy (rad) per cell can be used as a basis for comparison.

In contrast to systems in which the amount of a known chemical is tested for toxicity at different levels, the toxic agent or agents produced in irradiated media may be unknown. It does not necessarily follow that higher doses of radiation produce higher concentrations of the toxic compounds. For example, a toxic substance produced at low radiation doses may be radiosensitive and be destroyed by chemical interaction with radiolytic degradation products.

Since the nature of a presumptive mutagenic or cytotoxic agent may be unknown, it is desirable to concentrate these materials so as to magnify their responses in the biological tests. One approach employs a series of solvents of increasing polarity (Friedman et al., 1967). Some other approaches use ultrafiltration through graded membranes in order to concentrate extracts and pressings from the food or to remove water without the use of chemicals or heat.

MUTAGENIC AND CYTOTOXIC EFFECTS OF IRRADIATED MEDIA

Cytotoxic effects on non-mammalian cells in vitro

Taylor, Thomas & Brown (1933) reported that the growth of protozoa was inhibited when they were grown in a sterile culture medium previously irradiated with X-rays and Evans (1947) showed that when spermatozoa from sea-urchins (*Arbacia punctulata*) were first placed in irradiated (>100 000 R) sea-water and then used to inseminate fertile eggs of the same species, a reduction in survival time and a delay in first-cleavage cell divisions were observed in the fertilized eggs. The deleterious effects were attributed to hydrogen peroxide produced in the sea-water by the irradiation since the toxicity could be eliminated by adding catalase to the irradiated sea-water. The addition of hydrogen peroxide to non-irradiated sea-water in amounts equivalent to those produced by the irradiation caused similar toxic effects.

Heilbrunn & Young (1935) reported that the presence of ovarian tissue in an irradiated medium resulted in a more pronounced delay in cleavage of fertilized sea-urchin eggs than when the eggs alone were irradiated. The presence of other tissues did not induce a delay in cleavage and the investigators concluded that irradiation of ovarian tissue produced a substance or substances which acted on the eggs.

A toxic effect of irradiated medium on chick embryo cells was manifested by the inhibition of the growth of the cells and of their capacity to synthesize the Rous sarcoma virus (Levinson, 1966). The effect of the irradiation could be reproduced by adding hydrogen peroxide equal in concentration to that found in the irradiated medium. Catalase, added to distilled water before or after its irradiation, destroyed the biological effects of the irradiated water. The cytotoxic effect of the irradiated medium decreased with increasing cell concentration; for example, about half the cells were affected when plated at a concentration of 10^6 in a medium receiving 30 000 R, while only 1 % of the cells were affected when the cell concentration was reduced to 5×10^5 per plate.

Frey & Pollard (1966) reported that exposure of *E. coli* $15T^-L^-$, an auxotroph requiring both thymine and leucine for growth, to medium irradiated with gamma rays resulted in a cessation of DNA synthesis. The effect was eliminated by the addition of catalase to the medium. The degree of inhibition of cell growth depended on the radiation dose and could be simulated by addition of hydrogen peroxide in amounts equivalent to those formed by the irradiation. The bacterial cells reacted more vigorously with hydrogen peroxide from the medium than cells which were broken open; there was no effect on autoclaved cells.

Carbohydrate-containing media. Holsten, Sugii & Steward (1965) reported that a gamma-irradiated medium inhibited growth of carrot cells. From a systematic study of the effects of irradiation of the various components of the basal medium, it was revealed that the inhibitory effect was derived from

sucrose in the culture medium. The autoclaving of irradiated sucrose produced some enhancement of the biological effects. The same authors also showed that irradiated sucrose solutions inhibited mitosis in *Vicia faba* seedlings. The inhibitory effect of the irradiated, autoclaved sucrose was manifested even after prolonged storage, presumably at a low pH (3.3–3.5).

Holsten, Sugii & Steward (1965) related their findings to the safety of irradiated foods by stating that their work " . . . has other and obvious implications for the radiation sterilization of food, especially in those cases in which the material so irradiated is relatively rich in sugar. If radiation effects may be transmitted to cells via stable radiolysis products derived from sugar, one should clearly know whether or not these have biologically important consequences, both short and long term, before there is widespread use of radiation-sterilized foods that contain sugar ". It has been pointed out (Cook & Berry, 1966) that the manifestation of deleterious effects in *in vitro* tests does not necessarily mean that irradiated foods would prove to be deleterious *in vivo*, in view of detoxication mechanisms and the concentrations required to reach target organs.

Other studies have shown that irradiated sucrose affects the growth of plant cells such as those of the wild carrot (Bandiera, Morpurgo & Ricci, 1967). Irradiated, autoclaved sucrose solutions were toxic to paramecia (Joint Committee on Atomic Energy, 1968). The germination and growth of an ornamental plant, *Tropaeolum majus*, was inhibited by irradiated sucrose solutions (Kesavan & Swaminathan, 1967). The effect of storage on the irradiated medium and the cytotoxic effects depended on the radiation dose.

In studies on large populations of embryos of carrots and water-parsnips, grown with and without irradiated sucrose in the culture medium, the frequency of abnormal growth forms was increased in the presence of irradiated sucrose (Ammirato & Steward, 1969). The earlier the irradiated sucrose was applied during the course of development, the more frequent and extreme were the abnormalities. The irradiated sucrose appeared to act by causing certain, otherwise quiescent, cells to divide and to function as growth centres. Very low concentrations of irradiated sucrose appeared to stimulate cell division.

Blank (1935) reported that medium prepared from ultraviolet-irradiated agar, agar–water gels, or 20 different carbohydrates, inhibited the growth of *Bacillus subtilis*. The inhibitory effect was ascribed to the production of a diffusible, non-volatile, thermostable material (Blank & Kersten, 1935). Shortly afterwards, Baumgartner (1936) investigated the growth of *B. subtilis* and *Escherichia coli* when exposed to ultraviolet-irradiated media, including a 1% sucrose solution. He found that the irradiation of a 1%-sucrose solution or culture medium caused a drop in pH from 7.0 to 3.5–4.0 and the medium did not support growth. However, if the medium was neutralized before use, growth was not inhibited. If the sucrose solution was irradiated in the presence of excess calcium carbonate, thus preventing pH changes upon irradiation, no inhibition of growth was observed. It was claimed that about half the acidity formed by the irradiation of a 1%-sucrose solution was due to formic acid. Obviously, the pH should be readjusted to normal values before a biological assay is made.

In a review of his work on *E. coli*, Adler (1963) described medium effects involving catalase-negative and catalase-positive strains of *E. coli* designated *E. coli* H_{7-} and *E. coli* H_{7+}, respectively. He showed that a toxic material was produced in an irradiated suspension of *E. coli* H_7 which inactivated H_{7-}, but not H_{7+}, strains. The toxic material, identified as hydrogen peroxide, was destroyed by catalase but not by denatured catalase. Irradiated H_{7-} cells suspended in irradiated phosphate buffer or in phosphate buffer containing reagent hydrogen peroxide at the same concentration as in the irradiated buffer, were inactivated at the same rate. Interestingly, only irradiated catalase-negative cells were sensitive to low levels of hydrogen peroxide. Post-irradiation hydrogen-peroxide-sensitive states have been observed in other biological materials such as DNA (Conway, 1954) and bacteriophage (Alper, 1954).

The lethal effect of gamma-irradiated medium on *E. coli* was studied by Chopra (1969), using a strain (K12λs) which is sensitive to streptomycin. Presumably the medium was at or near neutral pH, and was irradiated under static conditions while saturated with air, but no details were given in the article. When *E. coli* was incubated in the gamma-irradiated minimal medium, the lethality increased as the radiation dose increased from 50 krad to 500 krad. The lethality depended on the number of bacteria per millilitre seeded into the irradiated medium, though the actual numbers of bacteria present were not stated. The component in the irradiated medium responsible for the lethality proved to be glucose.

The lethal effects could either be eliminated by catalase, or duplicated by the addition of hydrogen peroxide to the non-irradiated medium.

Other parameters studied by Chopra (1969) included dose-rate dependence. It was found that a medium given 200 krad of irradiation at a high dose rate (17 300 rad/min) was more lethal to *E. coli* than a medium incubated at a low dose rate (943 rad/min). The bactericidal activity of an irradiated medium decreased with storage time at 4°C but remained lethal after a month in the 200-krad- and 500-krad-irradiated media although it had nearly disappeared in the 100-krad-irradiated medium. It should be noted that the storage effects were observed in irradiated media whose pH was presumable at, or near, neutral.

Molin & Ehrenberg (1964) demonstrated that gamma-irradiated aqueous solutions of glucose (1%) strongly inhibited the survival of *Pseudomonas* sp. 128. The initial cell concentration was about 10^6/ml. The survival decreased as the radiation dose was varied from 0.03 Mrad to 10 Mrad. Bacteria previously exposed to the irradiated medium were more resistant to a subsequent exposure than bacteria exposed for the first time. This observation may be at least partly explained by the adaptation of bacteria to hydrogen peroxide (Watson & Schubert, 1969). Part of the antibacterial activity of the irradiated solution persisted after storage for 6 days. The antibacterial activity increased with decreasing pH of the irradiated solution and was abolished by the addition of catalase. Despite a high natural catalase activity, *Pseudomonas* sp. 128 was sensitive to hydrogen peroxide; as little as 1 μg/ml–5 μg/ml caused some mortality. Irradiated crystalline forms of glucose, which were subsequently incorporated into the growth medium, were not toxic except at very high radiation doses (20 Mrad).

The pH effect noted by Molin & Ehrenberg can be related to the fact that the production of hydrogen peroxide in aqueous solution increases with decreasing pH (Baxendale, 1964; Schubert, Watson & White, 1967). The concentration of hydrogen peroxide produced radiolytically is affected by many factors, including the presence of simple inorganic salts. For example, in studies on the effects of radiation on bacteriophage T1, Bachofer & Pollinger (1954) found that sodium nitrate protected the virus when it was irradiated in very dilute solutions. They were unable to explain the nature of the protective effect but it may have been due to a markedly lower production of radiolytic hydrogen peroxide. It has been observed (Barron, Seki & Johnson, 1952) that, compared with sodium chloride, small amounts of sodium nitrate strongly reduce the concentration of radiolytic hydrogen peroxide by a factor as high as 5.

The inhibition of growth of *Salmonella typhimurium* LT2 by irradiated sucrose solutions has been under systematic investigation (Schubert, 1967; Watson & Schubert, 1969; Schubert, Watson & White, 1967; Schubert & Watson, 1969a; Schubert & Watson, 1969b). In a gamma-irradiated (2 Mrad) aqueous 2% solution of sucrose, the antibacterial activity was greater when the solution was oxygenated during the irradiation. The antibacterial activity of irradiated solutions stored at the post-irradiation pH (about 3.3), had largely disappeared within a few days. The irradiated, oxygenated solutions retained their antibacterial activity when stored long enough for all detectable hydrogen peroxide to disappear. The oxygen-free irradiated sucrose solutions had little or no detectable hydrogen peroxide (<2 μg/ml) even immediately after irradiation. However, the inhibitory action could be enhanced by the addition of hydrogen peroxide at levels too low to be inhibitory by themselves.

It was concluded by Schubert, Watson & White (1967) that the cytotoxic action of irradiated sucrose was due, at least in part, to the formation of hydroxyalkyl peroxides (HAP) derived from the interaction of hydrogen peroxide with carbonyl compounds produced during the radiolysis of glucose and fructose in the low-pH solutions according to the following reactions (Hawkins, 1961):

$$\mathrm{RCHO + HOOH} \overset{\mathrm{H^+}}{\rightleftharpoons} \mathrm{RCH(OH)\text{-}O\text{-}O\text{-}H} \qquad (1)$$

$$\mathrm{RCH(OH)\text{-}OO\text{-}H + RCHO} \overset{\mathrm{H^+}}{\rightleftharpoons} \mathrm{RCH(OH)\text{-}O\text{-}O\text{-}CH(OH)R} \qquad (2)$$

The over-all reaction may therefore be represented as follows:

$$2\mathrm{RCHO} + \mathrm{H_2O_2} \overset{\mathrm{H^+}}{\rightleftharpoons} \mathrm{RCH(OH)\text{-}OO\text{-}CH(OH)R} \qquad (3)$$

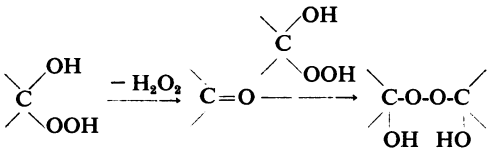
The loss of inhibitory activity in the irradiated solutions after storage at pH 7.0 was attributed to the hydrolysis of the HAP forming the corresponding organic acids, hydrogen and other products. The actual rate of hydrolysis and the products formed depend on the individual peroxide. Some of the pathways of hydrolysis can be described by the following reactions:

$$\mathrm{RCH(OH)\text{-}O\text{-}O\text{-}CH(OH)R} \xrightarrow{\mathrm{OH^-}} \begin{cases} 2\mathrm{RCHO} + \mathrm{H_2O_2} \\ \mathrm{RCH} + \mathrm{RCOOH} + \mathrm{H_2O} \\ 2\mathrm{RCOOH} + \mathrm{H_2} \end{cases} \quad (4)$$

The inhibitory action of irradiated sucrose could be simulated by mixtures of hydrogen peroxide with various aldehydes thought to be formed radiolytically, namely, glyoxal, glyceraldehyde, etc. (Schubert, Watson & White, 1967).

The addition of catalase prior to, or shortly after, inoculation of the irradiated sucrose eliminated most of the inhibitory activity produced by irradiated, oxygen-free sucrose solutions and only partly eliminated the inhibitory activity manifested by irradiated, oxygenated sucrose solutions (Schubert & Watson, 1969a). The effectiveness of catalase decreased when the time elapsing between inoculation and the addition of catalase was increased, but when the catalase was added before inoculation the effectiveness was independent of time. From considerations of mass action and other factors, e.g., the higher aldehyde concentrations, it was concluded that hydroperoxides predominated in irradiated, oxygenated sucrose solutions.

The effects of heating or autoclaving on the cytotoxic effects of irradiated sucrose solutions on *S. typhimurium* LT2 proved to be interesting (Schubert & Watson, 1969b). The inhibitory action was not modified when sucrose solutions were heated prior to irradiation but when irradiated sucrose solutions (pH 3–3.5) were subsequently heated, their antibacterial activity was considerably enhanced. The higher the temperature and the longer the duration of heating, the greater was the increase in antibacterial activity, at least in the temperature range 25°C–100°C and up to 4 hours heating; this was true for solutions irradiated in the presence of oxygen or in its absence. The lag, i.e., the period prior to exponential growth, in *S. typhimurium* LT2 was prolonged, for example, from 0.5 hour in an unheated, oxygen-free, irradiated, sucrose solution to 19 hours when the solution was boiled for 2 hours after being irradiated. The enhancement of antibacterial activity was interpreted as being due to hydroperoxides, ROOH, being converted by heating to, presumably, more inhibitory dialkyl peroxides, ROOR′, according to the following reactions (Mageli & Sheppard, 1967):



(5)

The antibacterial activity of heated, irradiated sucrose solution, as expected, was reduced less efficiently by catalase than an unheated, irradiated sucrose solution. It is known that heating may also produce polymeric peroxides (Mageli & Sheppard, 1967). In fact, the irradiation of oxygen-free sugars produces appreciable levels of polymeric compounds (Snell, 1965). Polymer formation is also a function of dose rate—another factor to be considered when comparing the results of different investigators.

The antibacterial activity of irradiated sucrose solutions is reduced or eliminated by histidine, histamine, glutamic acid and other nitrogenous compounds (Schubert & Watson, 1969b). The most effective compounds were histidine and histamine which were active against boiled, irradiated solutions, and, in all cases, were more effective than catalase. The antibacterial action of histidine will be discussed later, but is attributed to the fact that it is easier to rupture the O–O bond in organic peroxides than in hydrogen peroxide.

Media containing nitrogen compounds. Irradiation of nitrogenous compounds produces cytotoxic agents, the response depending on the particular compound and the test organism. When a dilute (e.g., 0.03M) oxygenated solution of histidine was irradiated, a marked inhibition of the growth of *S. typhimurium* LT2 is observed with as little as 25 krad (Schubert, Watson & Baecker, 1969). Even smaller radiation doses would have produced inhibition, especially if the number of cells per ml (10^7) had been reduced. The inhibitory activity can be eliminated by the addition of catalase to the irradiated medium prior to inoculation or by boiling.

The cytotoxic effect of the irradiated histidine solutions can be simulated by the addition of equivalent amounts of hydrogen peroxide to non-irradiated histidine. The irradiation of histidine is found to produce a histidine–peroxide adduct which was subsequently crystallized and found to behave in the same manner as a mixture of histidine and hydrogen peroxide (Schubert, unpublished data). The formation in biological media of hydrogen peroxide adducts with a variety of compounds—amino acids, carboxylic acids, carbonyl compounds, nucleic acid bases, etc.—is a general reaction which will be discussed later. It is apparent, therefore, that many of the reactions of irradiated media are presumably derived from, but are not due directly to, hydrogen peroxide.

Popov (1967) reported that gamma-irradiated solutions of pyrimidine bases were toxic to *Paramecium caudatum* (200 cells per ml). Irradiated thymidine

killed more infusoria than irradiated water or buffer and inhibited their division by 50%. The toxicity of the irradiated media was removed by the addition of catalase. Irradiated solutions of uridine or uracil were more toxic than irradiated thymidine.

Irradiated natural products. Cytotoxic effects have been observed in the juice extracted from pineapples immediately after gamma-irradiation with doses up to 500 krad (Makinen, Upadhya & Brewbaker, 1967). When onion roots were treated with pineapple juice adjusted to pH 7, mitotic frequencies were depressed by the juice from irradiated fruit. However, juice extracted from irradiated pineapples stored for 8 days at 18°C did not depress mitotic frequency, except at the higher radiation doses beginning at 100 krad.

Water extracted from irradiated leaves of young *Vicia faba* plants inhibited the growth of seedlings and the mitotic activity of roots submerged in these extracts (Kuzin, 1962b, p. 271). Experiments on extracts from the leaves of 9 different plants gave similar results and, in addition, these extracts inhibited the growth of *E. coli*. Further experiments demonstrating medium effects involving cytotoxic effects in a variety of cells and tissues are described by Kuzin (1962b).

Dupuy & Usciati (1966) studied the effects on the growth of *E. coli* in nutrient broth of extracts prepared from irradiated (10 krad–50 krad) potatoes and non-irradiated potatoes. While the extracts of both irradiated and non-irradiated potatoes inhibited bacterial growth, the extracts from irradiated potatoes were more inhibitory when tested under identical conditions. The inhibitory activity disappeared from both irradiated and non-irradiated extracts when the potatoes began to sprout.

Swaminathan, Chopra & Bhaskaran (1962) observed several cytological abnormalities during mitosis in the root tips of barley when embryos excised from non-irradiated mature seeds were grown on potato mash irradiated with 20 krad, 40 krad and 80 krad of X-rays. The potato pulp was prepared by mashing potatoes in a glass mortar and removing the expressed water. The pulp, having a moisture content of 70%, was spread evenly on Petri dishes and irradiated. Embryos from seeds of diploid and colchicine-induced autotetraploid barley were planted in the potato pulp immediately after irradiation of the pulp. The embryos were germinated on the potato pulp at 24°C and 50% humidity for 24 hours. The meristematic regions of the roots were used for preparing Feulgen squashes. Mitosis and root growth were inhibited dramatically in the embryos cultured in irradiated potato mash. No well-spread and clear metaphase plate was found in any of the numerous fixations.

Dharkar (1969) treated potatoes with different radiation doses (0 krad, 25 krad and 50 krad) and the tubers from each treatment were divided into 3 groups. The first group was used immediately after irradiation, the second and the third were used 24 hours and 48 hours after irradiation. The aqueous extract of these potatoes was clarified by centrifugation and filtration and thermally sterilized at 15 lbf/in^2 (1 kgf/cm^2) steam pressure for 15 minutes and were inoculated with 24-hour-old washed cells of *Serratia mescarahens*, *E. coli*, *Saccharomyces cerevesiae* or *Staphylococcus albus*. Germination of barley and maize seeds steeped at 10°C in clarified extracts of control potatoes or in extracts of irradiated potatoes made immediately after irradiation (25 krad and 50 krad) was followed every day by measuring the height of the seedlings.

The extract obtained from potatoes immediately after irradiation had a definite growth-depressing effect on all the 4 micro-organisms tested. Results of experiments carried out with extracts of irradiated tubers 24 hours after irradiation, however, did not show any difference in the growth patterns. In the studies carried out 48 hours after irradiation, a definite stimulatory effect of the irradiated media on the growth of all the organisms was observed; it was possibly due to a greater availability of amino acids and sugars because of radiolytic breakdown of protein and starch.

It would have been interesting if information had been available on (1) the pH of the extracts before and after irradiation and autoclaving and (2) whether the autoclaving of extracts from the irradiated potatoes modified the antibacterial action.

In the experiments with barley and maize seedlings, no observable difference in the germination of these seeds, soaked previously in either irradiated or control extracts, was seen.

Cytotoxic effects involving irradiated cytoplasm or nuclei of cells have been the subject of many investigations and reviews, e.g., Kuzin (1962a, chapter 7). For example, when amoebae irradiated supralethally with X-rays received enucleated portions of non-irradiated cells, the irradiated nuclei underwent mitosis and these amoebae produced mass culture (Daniels, 1959). In his classic work on ovarian eggs of salamanders and frogs, Duryee (1949) was able to produce typical nuclear radiation damage in

non-irradiated cells by microinjections of cytoplasmic material withdrawn from irradiated eggs. It was possible to produce chromosomal and nucleolar injuries by exposure of isolated normal nuclei to irradiated cytoplasmic brei. Duryee concluded that irradiation of the cytoplasm produced toxic materials that were transmitted into the nucleus in 10–30 minutes at 23°C.

Cytotoxic effects on mammalian cells in vitro

Irradiated solutions (1%) of glucose or fructose were shown by Berry, Hills & Trillwood (1965) to be toxic to mammalian cells. They used 2 lines of mammalian cells, a HeLa S-3_{oxf} aneuploid developed from a human cervical carcinoma and mouse strain L dermal fibroblast. When radiation-sterilized solutions of glucose or fructose were added to the growth medium, gross cytotoxicity was demonstrated; the average growth in 7 days was about 1% of that of the controls. The toxicity of the irradiated solutions was retained when stored up to 6 months at 5°C and also after autoclaving. As little as 50 krad produced cytotoxicity; a maximal effect was obtained after a dose of about 100 krad.

Autoclaved glucose and fructose solutions showed some cytotoxicity which decreased with storage. The observed cytotoxicity was attributed largely to glyoxal since the concentration of glyoxal in irradiated sugar solutions, as calculated from the data of Phillips (1961), produced a degree of cytotoxicity corresponding to that of the irradiated solutions. Actually, the concentration of glyoxal produced by irradiation is much less (Gotlieb & Markakis, 1968; Scherz et al., 1968) than that reported by Phillips. Consequently, the cytotoxicity could not have been caused by glyoxal alone. In their test system, Gotlieb & Markakis (1968) found that 5-hydroxymethyl furfural, which is one of the products formed from autoclaved glucose and fructose, was only mildly cytotoxic. In other work, Hills & Berry (1967) showed that the observed cytotoxicity was not due to formic or oxalic acids as had been suggested (Steward, Holsten & Sugii, 1967).

The growth of cells of a lymphoma strain, L5178Y, originally isolated from a DBA/2 mouse, was found to be inhibited by an irradiated growth medium containing 8% of foetal bovine serum (Scott, Diss & Sturrock, 1966). Most of the inhibitory effect disappeared when the irradiated medium (pH 7.8) was stored for 12 hours at 37°C before the inoculation of cells. The remarkable thing about this study was the low dose, 850 rad, required to inhibit cell multiplication. However, only 3000 cells/ml in the irradiated medium were added, so that the dose per cell was 850/3000 rad = 0.28 rad—a value actually similar to those obtained by investigators in related system using 10^2–10^6 times more cells but higher radiation doses.

A toxic effect on human fibroblasts was demonstrated by Kellner & Kaindl (1967) on a growth medium into which irradiated glucose solutions were incorporated. The fibroblasts were of different strains and were cultivated from explants using connective tissue and skin. The cell counts decreased with increasing radiation dose (0–1.44 Mrad). Maximum damage occurred with doses of 0.96 Mrad. It was postulated that at 1.44 Mrad, the intermediate toxic products were decomposed. Storage of the irradiated glucose solutions (presumably at low pH) for 6 weeks at 4°C did not result in any reduction of cytotoxicity. Non-irradiated glucose solutions boiled for 20 minutes or autoclaved at 120°C for 20 minutes were only slightly inhibitory.

Shaw & Hayes (1966) showed that irradiated sucrose solutions at concentrations of 0.55% were extremely toxic to human lymphocytes when added to the venous blood culture at the beginning of the culture period and treated for 72 hours. Mitoses were inhibited and many of the non-dividing cells appeared pycnotic and had not undergone normal " blast " transformation. Degenerated mitoses were observed and the chromosomes were severely damaged. The chromatin material was clumped or the chromosomes appeared shattered or pulverized.

Mutagenic effects on non-mammalian cells in vitro

Genetic damage to a large variety of cells grown in irradiated media has been demonstrated. The irradiated materials has consisted of sugars, foods, food components, amino acids, bacterial growth medium, etc. Excellent reviews of the literature have been given by Stone (1955), Scarascia-Mugnozza, Natarajan & Ehrenberg (1965), Muller (1954), Zelle & Hollaender (1955) and Wyss (1963), among others. In this section, therefore, attention is given to a selected number of reports which deal with the chemistry and biology of irradiated foods.

In many cases, the observed mutagenicity was related to the presence of hydrogen peroxide produced in the medium following irradiation with ultraviolet or ionizing radiation. However, it must be emphasized that it is often not the hydrogen peroxide as such which is responsible, but toxic compounds produced by the interaction of hydrogen

peroxide with one or more components already present in the substrate or produced by radiolysis, e.g., amino acids or carbonyl compounds (Schubert, Watson & Baecker, 1969).

In the pioneering work of Stone, Wyss & Haas (1947), cultures of *Staphylococcus aureus* were grown in broth irradiated with ultraviolet light. Bacteria grown in the irradiated broth developed resistance to penicillin and streptomycin and were true mutants. When a mineral-salt medium was irradiated, no increase in mutation rate was observed, but if amino acids and the vitamins thiamine, niacin, and biotin were added to the medium, a marked increase in mutation rate was observed. A marked toxic effect which manifested itself in the inhibition of growth was traced to the glucose in the medium. By irradiating the medium only to an extent that still permitted growth, the mutation rate of cultures grown in such media was not enhanced. Irradiation of the amino acids alone increased the mutation rate.

Stone, Wyss & Haas (1947) suggested that the natural radiation background may produce mutation by an indirect effect as well as by a direct hit on a gene, which is usually assumed. It is interesting to cite their conclusion: "Natural radiation is much more important in the mutation process if it can induce mutation by an effect on the food as well as in the organism".

In the follow-up to the above studies, Wyss et al. (1948) treated the nutrient broth with a chemical agent and let the mixture stand for 1 hour. If tests indicated that the added agent had disappeared, the medium was inoculated with about 10^6 cells/ml. After incubation, the assay for resistant mutants was made. When hydrogen peroxide was the added agent, no growth of *S. aureus* took place at a hydrogen peroxide concentration of 9 μg/ml. However, with 3 μg/l and 1 μg/ml of hydrogen peroxide, a definite increase in mutation rate expressed by penicillin or streptomycin resistance was observed even though no hydrogen peroxide was detectable by chemical tests on the broth at the time of inoculation. Agents such as chlorine, iodine, sodium nitrite and potassium permanganate did not increase the mutation rate.

The increase in mutation rate caused by hydrogen peroxide was not affected by the time elapsed between addition of hydrogen peroxide and inoculation for periods of 15 minutes to 22 hours. Consequently, it was concluded that the effect of hydrogen peroxide was due to its reaction with some component in the medium. Wyss et al. (1948) were able to enhance the mutation rate by simply adding mixtures containing hydrogen peroxide and various compounds such as phenyl alanine, tryptophane, tyrosine, adenine, uracil and guanine.

All the above-mentioned mutagenic effects were eliminated by the addition of catalase to the medium (Wyss et al., 1948). It was suggested that the catalase decomposed not only hydrogen peroxide but also organic peroxides present in the broth. It was also assumed that the bacterial catalase decomposed hydrogen peroxide, as evidenced by the fact that the size of the inoculum was a factor in determining the magnitude of the mutation rate. Thus, with an inoculum of 10^7 cells/ml, no increase in mutation rate was observed as was the case with inocula of 10^6 and 10^5 cells/ml. Further, the addition of a catalase inhibitor, sodium azide, to the medium resulted in a marked increase in the mutation rate.

When hydrogen peroxide was tested in washed cells of *S. aureus* at levels high enough to kill most of the cells, the mutation rate of the survivors was the same as that of the controls. If the survivors were inoculated into non-irradiated nutrient broth and allowed to grow, the resulting culture showed no enhancement of the mutation rate. Thus, the effects observed with hydrogen peroxide had to be produced by organic peroxides produced by the action of hydrogen peroxide on the components in the medium. This is the same conclusion reached in studies on the inhibition of growth of *S. typhimurium* by irradiated sucrose or histidine (Schubert, Watson & White, 1967; Schubert, Watson & Baecker, 1969).

Studies on Neurospora. The work of Stone and of Wyss was confirmed by Wagner et al. (1950) using wild-type strains of *Neurospora crassa*. They produced biochemical mutations at a rate significantly higher than the controls by treating the conidia with nutrient broth previously treated with ultraviolet light, and also by treating conidia directly with hydrogen peroxide and potassium cyanide. Mutations were detected after being passed through the sexual cycle. The mutation rates obtained indirectly were lower than the rates induced by direct irradiation. It was also observed that the mutation rate was related to the concentration of conidia, being higher at lower concentrations.

Previously, Dickey, Cleland & Lotz (1949) demonstrated that mixtures of hydrogen peroxide with carbonyl compounds, e.g., hydrogen peroxide plus formaldehyde or hydrogen peroxide plus acetone were highly mutagenic to *Neurospora*, in that they caused reverse mutations at an adenine-less locus.

The corresponding concentrations of hydrogen peroxide alone or carbonyl compound alone were only weakly mutagenic in the particular test system employed. Several organic peroxides proved highly mutagenic, e.g., *tert*-butyl hydroperoxide, and hydroxymethyl *tert*-butyl peroxide. Ionizing and ultraviolet radiations also induced mutagens following direct irradiation. Treatment of the spores with irradiated medium also produced mutations with about the same frequency as those observed after treatment with pure organic peroxides (Dickey, 1950).

Studies on medium effects were made by Jensen et al. (1951) using the *Neurospora* back-mutation technique with an adenine-less strain. They observed a rather strong mutagenic effect which appeared to result from the reaction of hydrogen peroxide, or its decomposition products, with cell components. The mutation rate, but not the toxicity of hydrogen peroxide, was stronger in daylight than in the dark, which suggested that oxygen atoms or hydroxyl radicals were involved. The addition of the antioxidant, nordihydroguaiaretic acid (NDGA), inhibited the mutagenic action of hydrogen peroxide, but was less effective on a mixture of hydrogen peroxide and formaldehyde. Irradiation with ultraviolet light of a medium containing formaldehyde before the addition of conidia greatly increased the mutation rate in the *Neurospora*. It was suggested that the treatments were mutagenic because free radicals, formed by decomposition, reacted with molecules within, or near, the gene.

In later work by Malling et al. (1959), the effect of combined treatment with ultraviolet light and formaldehyde or hydrogen peroxide on the reverse mutation rate of 2 alleles of *N. crassa* was investigated. The reverse mutation rate of the adenine-less mutant was greatly enhanced by combining ultraviolet radiation with pre- or post-treatment with both chemicals, whereas the reverse mutation rate of the inositol-less mutant may have been slightly decreased. It was suggested that the former effect was a result of indirect action and the latter effect a direct action of radiation.

Carbohydrate solutions. Holsten, Sugii & Steward (1965) tested the effects of irradiated sucrose incorporated into basal medium on the chromosomes of buds of *Tradescantia* microspores and roots of young *Vicia faba* seedlings. In both cases, chromosomal aberrations were found which were similar to those attributable to direct radiation of the tissue.

Lateral roots of *Vicia faba* were treated with gamma-irradiated glucose solutions (Ma, 1968). Solutions of glucose (20%) were irradiated with 0.5 Mrad, 1.0 Mrad and 2.0 Mrad, respectively; some of the solutions were diluted with spring water to 2% and 4% immediately after irradiation; others were aged 92 hours before dilution. Altogether, 50 metaphases were examined on each of 4 slides of every fixation group and chromosomal aberrations were found to be almost exclusively centromere breaks or breaks at the secondary constriction of the " M " chromosome (metacentric chromosome with secondary constriction). No significant differences in the number of deletions or interchanges were found between the controls and cells grown in irradiated glucose. It was concluded that the irradiated glucose solutions did not induce true chromosome aberrations but a weakening of the centromere.

It has been suggested that the cause of mutagenic effects in *Vicia faba* root tip is due to the low pH of the irradiated solutions (Bradley, Hall & Trebilock, 1968). Test solutions of irradiated (2 Mrad) sucrose solutions were adjusted to form a series from pH 3.0 to pH 7.5. It was noted that the root hairs affected the pH of the solutions so that average readings before and after treatment were recorded. When the pH values were lower than 4.6, the proportion of abnormal anaphases in root tips exposed to either control or irradiated solutions increased with decreasing pH. The degree of increase of aberrant anaphases was, however, higher in irradiated solutions. When all sucrose solutions were adjusted to pH 6.5 before treatment, no differences in proportion of abnormal anaphases were noted between the control and irradiated solutions. The types of chromosome aberrations noted were the same for control and irradiated solutions. Also, the proportions of specific types of aberration (spindle defects, fragments and bridges) were almost identical.

Moutschen & Matagne (1965) treated onion and barley root-tip cells with a solution of gamma-irradiated crystalline glucose. The glucose solutions were prepared by irradiating crystalline glucose monohydrate with doses ranging from 0.05 Mrad to 79 Mrad. The irradiated solid glucose was dissolved in deionized, sterile water to give a 1%–2% solution. An increase in chromosomal aberrations—mainly of isolocus breaks, usually localized at the centromere—was found. However, no radiation-dose effect was found for the dose range investigated, though most of the results cited were for radiation doses of 48 Mrad and higher. The increased number of chromosomal abnormalities, e.g., 5%, relative to

the controls (0.74%) was statistically significant.

Irradiated (2 Mrad), autoclaved sucrose produced no detectable levels of reversion from auxtrophy to prototrophy (tryptophane) when *E. coli* strain WP-2 was grown in media into which irradiated sucrose was incorporated (Joint Committee on Atomic Energy, 1968, p. 120).

No mutations were found in histidine-deficient strains of *S. typhimurium* following incorporation of irradiated sucrose (Watson & Schubert, unpublished data). However, a negative result in a reverse mutation study does not indicate lack of mutagenicity.

Exposure of paramecia during the early interphase to irradiated sucrose did not produce any recessive lethal mutations as expressed in terms of the percentage of the exautogamous isolates that died (Joint Committee on Atomic Energy, 1968, p. 120). It is interesting to note that exposure of paramecia to hydrogen peroxide does not produce mutagenesis (Kimball, Hearon & Gaither, 1955). Negative results in a single test system as described here could be due to many factors such as the large numbers of cells needed for the tests or to physical or mechanical parameters such as the thickness of the cytoplasmic layers in the paramecia.

Natural products. The effect of irradiated fruit juices on germinating seeds of barley and onion was investigated by Chopra, Natarajan & Swaminathan (1963). They used orange and apple juices irradiated in glass vials with 200 krad—a suggested pasteurizing dose.

In the cytological studies, root tips of barley and onion were submerged in irradiated and non-irradiated fruit juice for varying periods from 4 to 24 hours. Subsequently, the seeds were examined for chromosomal aberrations by standard procedures. When chromosome breakage was induced with fractionated treatment (8 hours for 3 consecutive days) about 20% of the chromosome breaks occurred at the region of the centromere, a characteristic effect of some alkylating agents. There were very few chromatid aberrations. The growth of roots and shoots was stimulated in the 4-hour treatment and was retarded in the 24-hour treatment.

The pH of the irradiated fruit juices dropped slightly after irradiation; orange juice from 4.35 to 4.30, commercial apple juice from 3.37 to 3.23, and fresh apple juice from 4.25 to 4.22. An increase in peroxide content, as measured by an iodometric method, was found following irradiation of values ranging from 2000 μg/ml to 5000 μg/ml. Such high values seem to be an order of magnitude too high (see, for example, Schubert, Watson & White, 1967).

The cytotoxic effects of extracts from gamma-irradiated pineapple juice (Makinen, Upadhya & Brewbaker, 1967) were described earlier (p. 880). In the same study, the effects of irradiated pineapple on the frequency and types of chromosome breaks in onion root cells were also investigated; 300 cells were examined for each treatment. When root tips were exposed to pineapple juice immediately after it had been irradiated, there was a significant increase in the frequency of breaks (almost all at the centromere) but no correlation with dose (from 30 krad to 500 krad).

When the irradiated extracts were stored for 8 days, only the higher doses, 100 krad and 500 krad, produced breaks, indicating that some detoxication occurred in the juice receiving 30 krad and 50 krad. The authors suggested that the higher doses impaired enzymes which participated in the detoxication.

Kuzin (1962b) subjected the leaves of 14-day-old *Vicia faba* plants with up to 5 krad–20 krad of gamma irradiation. Subsequently, the irradiated leaves were frozen, triturated and extracted with water. The water extract was sterilized by boiling; the precipitated proteins were removed and the filtrate was tested for cytological effects on the roots of 4-day-old seedlings of *Vicia faba*. Apart from mitotic inhibition, the extracts produced a 6-fold increase of chromosomal aberrations over that of extracts prepared from non-irradiated leaves.

In their studies on the cytotoxic effects of extracts of irradiated potatoes (p. 880), Swaminathan, Chopra & Bhaskaran (1962) found that abnormalities in the embryonic cells from barley root tips included chromosome stickiness at both metaphase and anaphase, lagging chromosomes at anaphase, irregular shape of nuclei, weak-staining and disintegration of nuclei, formation of cells with micronuclei of varying sizes and numbers, and the presence of binucleate cells and cytoplasmic basophilia.

The frequency of cells with and without distinct micronuclei was determined. It was found that (1) the frequency of cells with micronuclei was greatly increased in barley embryos cultured on irradiated potato mash; (2) the number of cells with micronuclei increased with increasing X-ray dose; (3) the increase in the frequency of aberrant cells was more marked in diploid barley than in autotetraploid; and (4) the frequency of cells with micronuclei was significantly reduced in diploid bar-

ley when the potato medium containing the embryos was transferred to an oxygen atmosphere immediately after irradiation.

There is little doubt, as shown by the occurrence of minute micronuclei, that chromosome breakage had been induced. However, the difficulty of obtaining well-spread metaphase plates precluded any quantitative evaluation. The reduction in cytological aberrations produced by post-irradiation exposure to oxygen is difficult to interpret without further experimentation. It is conceivable, however, that oxygen " scavenged " free radicals or prevented polymerization reactions that would lead to the formation of cytotoxic substances. It would be of interest to ascertain whether the addition of catalase before or after irradiation would have modified the cytotoxicity of the irradiated potato pulp.

In a follow-up to the studies mentioned above, Chopra & Swaminathan (1963) irradiated potatoes with 20 krad and stored them in the cold (2°C–3°C) with the controls. After storage for about 8 months, the potatoes were kept in the open in the laboratory. Sprouting was normal in the non-irradiated tubers but was completely inhibited in the irradiated ones. The 2 sets of tubers were then used for the preparation of mash on which germinating barley embryos were planted. In root meristems derived from embryos grown on mash from normal potatoes, 9 cells had micronuclei out of a total of 3022 cells examined. However, in root tips from material cultured on mash from irradiated potatoes, the number of cells containing micronuclei was 64 out of 2811 cells studied. Thus there was about an 8-fold increase in the frequency of occurrence of cells with micronuclei, even after prolonged storage of the irradiated potatoes.

Mutagenic effects on mammalian cells in vitro

The first studies in which the induction of chromosomal aberrations in mammalian cells *in vitro* was seen were reported by Shaw & Hayes (1966). The test solutions were irradiated (2 Mrad) autoclaved solutions of sucrose supplied by Holsten, Sugii & Steward (1965), some of whose results were described earlier (pp. 876-877). The cytotoxic findings of Shaw & Hayes have been described on page 881.

Sucrose solutions were added 24 hours before the cultures were collected. Cultures of human lymphocytes containing irradiated or non-irradiated sucrose at 8 different concentrations were prepared. Concentrations of sucrose of 0.36% or higher in the culture media reduced the mitotic rate. At all concentrations of irradiated sucrose tested (0.91%–0.009%) there was an increase in both the number of breaks and in the proportion of mitoses with 1 or more breaks. The proportion of breaks which were of isochromatid type was much higher in the treated cells (52/234) than in the control cells (2/37). Among the treated cells there were 8 double fragments and 2 single fragments, compared with 1 single fragment in the controls. Dicentric chromosomes and rings were not observed. Altogether, 9 exchange figures were counted among the treated cells.

The observations of Shaw & Hayes (1966) were duplicated as closely as possible in work done at the Oak Ridge National Laboratory, USA (Joint Committee on Atomic Energy, 1968, p. 118). Irradiated, autoclaved sucrose produced large numbers of aberrations as reported (Shaw & Hayes, 1966). Interestingly, non-irradiated or irradiated solutions that were not autoclaved did not produce a significant increase in aberrations.

Kesavan & Swaminathan (1966) reported that gamma-irradiated (0.10 Mrad–0.50 Mrad) TC-199 culture medium produced breakage of chromosomes in human leucocytes. The prominent effects observed, apart from the considerable inhibition of mitosis, included many gaps, breaks and fragmentations of chromosomes. When the irradiated TC-199 medium was stored for 15 days, the cytotoxic properties persisted.

Plasma from human patients who were exposed to ionizing radiation produced chromosomal breaks in normal, non-irradiated lymphocytes in short-term cell culture (Hollowell & Littlefield, 1968; Goh & Sumner, 1968). Hollowell & Littlefield (1968) used cell-free plasma from 6 patients with tumours which had been treated with X-rays. For controls they used autologous or non-irradiated homologous plasma (" normal plasma ") and plasma from 6 patients with comparable tumours who had not yet received radiation therapy (" non-irradiated plasma ").

The 2 control groups showed no significant differences in the frequency of chromatid breaks (3.6/100 and 4.2/100 cells from " normal " and " non-irradiated " plasma, respectively). However, in the irradiated plasma, the frequency of breaks was 10.7/100 cells. The frequency of chromosome breaks was 7.1/100 cells in the irradiated plasma compared with 0.4–0.9/100 cells in the controls.

In control groups, only 1 transformed chromosome was seen, an exchange figure, in a culture with autologous plasma. In the irradiated plasma

group, 3 dicentric chromosomes, 1 ring chromosome, and 1 quadriradial exchange figure were seen. Excluding gaps, 16.9% of the cells in the irradiated plasma group contained abnormal chromosomes, compared with 3.9% in the non-irradiated group and 4.1% in the normal plasma group. The differences between the irradiated plasma group and either of the other groups were statistically significant. Many of the cells treated with irradiated plasma had clumped chromosomes and stranding among the chromosomes. This was rarely seen in the control groups.

Altogether, 3 of the non-irradiated tumour patients were studied again after 2 of them had received X-ray doses of 1000 R and 1 had received 3000 R. Excluding gaps, there was a significant increase in the frequency of cells having aberrant chromosomes after the patient had been X-rayed (4.2% of cells had aberrant chromosomes before treatment; 18.9% of cells in the post-treatment group). Transformed cells were seen in the post-irradiation group.

Leucocytes from normal persons were cultured with autologous or homologous plasma and with plasma from a group of patients who had been accidentally exposed 7 years previously to total-body, mixed, gamma-neutral irradiation (Goh & Sumner, 1968). The same authors also found that the plasma from these patients after irradiation treatment was capable of inducing chromosomal breakage in normal leucocytes. Altogether, 114 (8.7%) chromosomal breaks were found in 1318 metaphases obtained from normal peripheral blood leucocytes cultured with plasma obtained from the irradiated patients while only 1% of chromosome breaks were found in the control groups. The nature of the humoral factor is unknown. It has been speculated that it may be a virus or an enzyme.

Mutagenic and cytotoxic effects induced in vivo

Relatively few experiments have been carried out in which the irradiated food or medium has been administered by injection or feeding. Experiments with mammals, in particular, have been too few for general conclusions to be drawn at this time other than to indicate the necessity for further and more complete experiments.

Drosophila. Because of well-established techniques for the quantitative detection of mutation frequencies, *Drosophila* has been widely used for studies of mutagenic action. The effects of feeding irradiated medium to *Drosophila* have been reviewed (Scarascia-Mugnozza, Natarajan & Ehrenberg, 1965; Rinehart & Ratty, 1967). The results have appeared contradictory, though some of the negative findings did not include the possible influence of gonial mutations. Swaminathan et al. (1963) reported an increase in dominant and sex-linked recessive lethality and an increase in the frequency of visible mutations in *Drosophila* fed irradiated food. Hossain, Mollah & Malik (1967) found no toxic or mutagenic effects in *Drosophila* fed irradiated banana (35 krad–55 krad).

Rinehart & Ratty (1967) measured the frequencies of sex-linked recessive mutations in germ cells for *Drosophila* males cultured on aged or non-aged, irradiated (0.15 Mrad and 3 Mrad) or non-irradiated whole food or food supplements. The experiments showed that irradiated whole food was mutagenic. No change in the results was found when the treated food or food components was stored for 3 weeks before use.

The findings of Rinehart & Ratty (1967) support the findings of other investigators, such as those of Chopra (1965) and Khan & Alderson (1965), for example. Whether these results are applicable to man is, of course, problematical.

Munoz & Barnett (1968) injected irradiated (2 Mrad–5 Mrad) solutions of fructose and glucose into *Drosophila.* When sex-linked recessive lethals were counted in spermatozoa and spermatogonia, a statistically significant increase in the mutation frequency in spermatogonia was observed.

Mammals. The effect of long-term feeding of irradiated rat food, as well as derived distillates and water extracts, on the lymphocyte number of the peripheral blood was studied by Ehrenberg, Löfroth & Ehrenberg (1965) and Löfroth et al. (1966). They used male Sprague–Dawley rats whose ages varied from 4 to 16 months at the beginning of the experiment. The rat food—hard dried cakes—was gamma-irradiated at a dose rate of 0.4 Mrad–0.5 Mrad per hour and with total doses of 3 Mrad–9 Mrad, usually the latter. The rats fed irradiated food were said to show a decrease in the absolute lymphocyte numbers of 15%–20% within a few days to a few weeks after feeding began.

Distillates and water extracts prepared from the irradiated food (6 Mrad) caused a 15%–20% decrease in absolute lymphocyte numbers when given in the drinking-water, while corresponding distillates from non-irradiated food did not produce lymphopenia. The food which had been used for preparing the distillates—presumably the residue—was fed to rats, but the results were inconclusive.

The distillates from irradiated food were bactericidal when *E. coli* (sd-4) was used as the test organism (Löfroth et al., 1966). Some of the effect appeared to be due to the acids in the distillate, though at pH 5.5 little difference was observed in the antibacterial action of distillates from non-irradiated food compared with irradiated food.

The dominant lethal mutation test was applied in preliminary experiments carried out at the Oak Ridge National Laboratory, USA. Mice were inoculated (intraperitoneally) with a relatively large volume of irradiated sucrose solution prepared in various ways. Neither non-irradiated, autoclaved, nor irradiated, autoclaved sucrose solutions induced a statistically significant number of dominant lethals (Joint Committee on Atomic Energy, 1968, p. 120).

Short-term feeding studies were carried out by De, Aiyar & Sreenivasan (1969) in which weaning male rats were fed 2 ml of 10% sucrose solution, previously irradiated with 0.5 Mrad, twice daily for 8 weeks. A solution of ^{14}C-labelled sucrose which had been shown to have adverse biochemical effects *in vitro*, was also fed. The results of the feeding experiments were negative, i.e., no differences were found between the controls and animals fed irradiated sucrose solution or the labelled toxic fraction. The criteria employed included specific organ weights, histopathology of liver, kidney and spleen, and testes, maturity stages of sperm cells of testes, glycogen distribution in liver cells, liver lipid content, succinate oxidation, coupled phosphorylation in liver mitochondria, as well as acetate-1,2-^{14}C and leucine-1-^{14}C incorporation into lipids and proteins, respectively, by liver slices.

Rats and mice were fed by intubation for a period of 5 days, 3 times a day, with 2% solutions of irradiated (2 Mrad) sucrose prepared in different ways, i.e., with and without autoclaving, before and after concentration to 20%, with and without neutralization. No significant increase in chromosomal aberrations was found in the bone marrow cells even though such aberrations were produced *in vitro* (Schubert, Pan & Wald, unpublished data). Apparently, the chromosome-damaging substances were destroyed metabolically. These studies are still in progress.

A significant increase in chromosomal aberrations of meiotic cells in mice fed irradiated wheat flour was reported by Bugyaki et al. (1968). In their experiments, a selective strain of mice of C57BL stock was used because this strain shows an extremely low incidence of spontaneous translocations and other chromosomal aberrations as checked by extensive cytological examinations made over a period of several years.

Initially, 10 adult females and 2 male mice were given a diet which included 50% of irradiated wheat meal while a corresponding control group was fed the same diet but with non-irradiated wheat meal. The offspring furnished the animals for the experiment, namely, 50 females and 16 males for each group. The experiment covered an entire generation of mice.

The diet consisted of a basic food containing all the essential nutrients for normal growth and development of the mice. In the basic diet was incorporated an equal amount of non-irradiated or gamma-irradiated (5 Mrad) wheat meal. The meal was Belgian wheat, milled and sifted to 70% extraction. Each week, freshly irradiated meal was used.

Detailed cytogenetic examinations were made from spermatogenic cell preparations obtained from the testes by standard techniques. Several types of chromosomal aberrations were observed; at metaphase I: translocation patterns, rings and chains, fragments; at anaphase I: chromosome and chromatid bridges, free and attached fragments; at anaphase II and at gonial anaphases: bridges and fragments. Statistical analyses of the frequency of chromosomal aberrations were made for mice fed with irradiated wheat compared with the controls. The proportion of damaged cells in the treated animals was much greater than in the control animals.

To what extent these results have a bearing on radiation hazards to humans cannot be answered without further work. In any event, these experiments should be repeated at different dose levels. The radiation dose employed, 5 Mrad, was about 100–200 times greater than would be employed for the control of infestation in wheat. Further, no one would consume during his lifetime freshly irradiated, uncooked flour as half the total diet. Another consideration is the practical difficulty of avoiding artefacts in the preparation of slides of meiotic germ cells, in particular, as well as subjective bias in their interpretation. The microlesions which must have been produced in the chromosomes could be of even more serious consequence. Further, the hereditary implications of the data may be more pronounced when it is considered that the mature gamete is generally more sensitive to mutagens than the spermatogonia.

ORGANIC PEROXIDES

Inasmuch as organic peroxides have often been implicated as one of the chemical mutagens produced by irradiation, it is of interest to review a few of the investigations bearing on the subject (see p. 878).

Since it had been suggested (Dickey, Cleland & Lotz, 1949; Jensen et al., 1951; Sobels, 1963) that the mutagenic action of formaldehyde was due to the formation of an organic peroxide, $CH_2(OH)$-OO-$(OH)CH_2$, Sobels (1963), using a synthesized sample of the peroxide, injected an aqueous solution into the abdominal cavity of wild-type Oregon-K male *Drosophila*. The treated flies were afterwards tested for the incidence of sex-linked lethal genes. It was found that the dihydroxydimethyl peroxide induced mutations in mature sperm and in earlier stages of spermatogenesis. Pretreatment of the flies with the organic peroxide prior to irradiation enhanced the mutagenic effect of X-rays during the sensitive stage, while the mutagenic effect of the peroxide itself was exerted at some earlier stage.

Organic peroxides can inhibit the growth of ascites tumours *in vivo*, although the effectiveness varies with the structure of the peroxide (Weitzel, Buddecke & Schneider, 1961; Weitzel et al., 1961, 1963). Following its intraperitoneal inoculation in mice, the formaldehyde peroxide, dihydroxydimethyl peroxide, described above, completely stops the growth of Ehrlich ascites tumours and causes their degeneration (Weitzel et al., 1961).

Latarjet (1956) demonstrated that different bacterial strains of *E. coli* were inactivated by organic peroxides (succinic peroxide and cumene hydroperoxide) and that the sensitivities of the bacteria were of the same order as their sensitivities to radiation. The bacteria were more sensitive in the growing than in the resting state. The rate of inactivation increased with temperature but decreased when the bacterial concentration was increased.

Histidine peroxide, which is formed when an oxygenated solution of histidine is irradiated (Schubert, Watson & Baecker, 1969), delays the growth of ascites, decreases the cell count and prolongs the life of the animal (Weitzel et al., 1961). The antitumour effects might have been greater if an excess of histidine had been present in the injection solution to prevent dissociation of the adduct.

The effects of different organic peroxides on Walker tumours in rats, including those formed from aldehydes and ketones, were tested by E. V. Jensen (Dickey, 1950, p. 152). The peroxides were administered subcutaneously and showed no effect. Jensen suggested that an effect might have been obtained if the peroxides had been given intravenously.

CHEMICAL CHANGES INDUCED IN IRRADIATED MEDIA

Since water is a major component of most food, the chemical transformations induced by the passage of ionizing radiation closely relate to the radiation chemistry of water and aqueous solutions. A great deal is known about the general nature of the primary chemical transformations induced by irradiation of water and aqueous solutions. In the following paragraphs, only selected aspects of the radiation chemical reactions taking place in foods and food components are discussed. With respect to food components and model systems, a good deal of background information and experimental data are cited in books and review articles (Spinks & Woods, 1964; Allen, 1961; Glassner & Hart, 1964; Swallow, 1960; United Nations, 1958; *Advanc. Chem. Ser.*, 1965, 1968; Hart & Platzman, 1961; Phillips, 1961; Garrison, 1968; Liebster & Kopoldová, 1964; Alexander & Lett, 1967; Scholes, 1963; Weiss, 1964). Radiation chemical changes produced in different irradiated foods are described in various chapters and sections in several publications (Joint Committee on Atomic Energy, 1965, 1968; International Symposium on Food Irradiation, 1966; Wick et al., 1961; Gould, 1967; Metlitskii, Rogachev & Krushchev, 1967).

The chemical reactions which take place in irradiated media or food are initiated by (1) indirect action of the radiation-induced products of water decomposition producing (a) 3 primary radicals: the hydroxyl radical, OH; the hydrated electron, e^-_{aq}; and the hydrogen atom, H; and (b) 3 molecular products : hydrogen gas, hydrogen peroxide, and the hydrogen ion; and (2) direct action in which chemical bonds are ruptured by the dissipation of the energy from ionizing radiation within the molecules or atoms through which the ionization tracks pass, or by energy transfer from excited water molecules.

The chemical transformations which ensue depend on the rate at which the radicals react with various components in the media, and the reactions of the products of these interactions with each other and with the original components of the media to yield finally the stable end products, including polymerized and depolymerized compounds. Additionally, the

onized or excited components may undergo dissociation to produce free radicals; in the presence of oxygen, these radicals (e.g., R·) can form organic peroxy-radicals which may initiate a chain reaction in which hydrogen is abstracted from an organic molecule, RH, to yield an organic hydroperoxide, ROOH and another R· radical.

Many radicals react preferentially, that is, more rapidly, with certain molecules, referred to as " scavenging agents ", so that the normal reaction of a radical may not be manifested. Therefore, it is possible to modify deliberately the course of a radiation-induced reaction by adding scavenging agents. In materials as complex as food substances, in which innumerable scavenging sites are present, it is nearly impossible to predict, in any quantitative fashion, the distribution and kinds of molecules that may be produced, and to identify unambiguously or predict the many secondary products which result. In this respect, it is well to distinguish between the volatile products which are formed upon irradiation and the new products which remain behind. Reasonable reaction mechanism schemes are available with which to interpret the origin of the volatile products. However, from the standpoint of public health safety, the decisive consideration is the chemical composition of the residue or consumed portion of irradiated food. At the present time, it is apparent that the safety of irradiated food to the consumer must be evaluated by biological assay rather than in terms of known concentration of identifiable compounds. In this sense, the assessment of possible hazards from irradiated foods differs from that involved in studies on the toxicity of food additives, pesticides, drugs, etc., in which different levels of known amounts of these substances can be tested.

Radiation chemical studies on model systems have provided information which serve as a basis for predicting some of the reactions which take place in irradiated food. In an oxygenated, neutral, aqueous solution, for example, the yield of hydrogen peroxide becomes independent of radiation dose above about 200 krad. However, in acid solution, the yield of hydrogen peroxide increases with increasing radiation dose. The reasons lie in the fact that in neutral or basic solutions, hydrated electrons are the predominant reducing species while in acid solutions the hydrogen atoms predominate. In neutral solution, the breakdown of hydrogen peroxide by reaction with e^-_{aq} is sufficiently rapid to counteract its formation. Consequently, it is well to recognize that in secondary reactions involving hydrogen peroxide, the irradiation of an acid medium, e.g., a fruit juice, may be quite different from a medium that is neutral or one that has been neutralized.

Reactions involving the OH radical are independent of oxygen while the presence or absence of oxygen makes a great difference in reactions involving the hydrogen atom or e^-_{aq} since these are scavenged by oxygen. Further, the subsequent reactions of radicals with each other are greatly modified by the presence of oxygen. In irradiated, oxygenated solutions, for example, little polymer formation is observed whereas in irradiated, oxygen-free solutions of sugars, amino acids, and hydroxy acids, considerable polymer formation occurs (Snell, 1965; Barker et al., 1959). Further, the actual chemical compounds produced, and their yields, may differ considerably depending on the presence or absence of oxygen. For example, radiolysis of an aqueous solution of ethanol yields aldehyde as the main product in the presence of oxygen, and glycol in its absence. Similarly, glycerophosphate yields organic labile phosphate in the presence of oxygen and inorganic phosphate in its absence.

Carbohydrates

The radiolysis of simple sugars has been extensively studied, especially by Phillips (1961). It is of particular interest to note that yields are highly dependent on concentration. In a 5×10^{-4}M solution of glucose, for example, the chain scission reactions which yield aldehydic fragments are absent and the over-all breakdown of the glucose is less. The greater decomposition of D-glucose at a higher concentration, 5×10^{-2}M, appears to be due to direct energy transfer from excited water molecules. Abstraction by OH^- radicals appears to be the dominant process leading to the degradation of D-glucose at 5×10^{-4}M, but above 5×10^{-3}M, direct carbon–carbon scission occurs. No significant reaction between e^-_{aq} and glucose takes place.

Irradiation of sugars in the absence of oxygen gives rise to about the same degree of decomposition. However, an important, and perhaps most significant, difference from the biological standpoint is the fact that polymer formation occurs to a much greater extent in oxygen-free irradiations (Snell, 1965; Barker et al., 1959). The main route for polymer formation from glucose is believed to be through gluconic acid since the yield of gluconic acid from glucose solutions irradiated *in vacuo* is increased greatly. Further, there is a strong similarity in the

polymers obtained from 1 : 4-gluconolactone and those from glucose. Heating at 100°C for 10 minutes increases polymer formation as measured by the absorption of ultraviolet light at 260 mμ–270 mμ.

Scherz (1968) has given indirect evidence for the production of deoxy sugars expressed as 2-deoxyglucose in irradiated, oxygen-free, 1% solutions of D(+)-glucose and D(+)-sucrose.

The aldehydes formed from irradiated sugars can react with the hydrogen peroxide produced when oxygenated solutions of sugars are irradiated. The reaction products may include hydroxyalkyl peroxides which are antibacterial (Schubert, Watson & White, 1967). It is also possible that polymeric peroxides (Mageli & Sheppard, 1967) are formed. The existence of organic peroxides in irradiated sugars is shown by the fact that a substantial part of the antibacterial action is eliminated by catalase (Schubert & Watson, 1969). Many hydroxyaldehydes—glycolaldehydes, glyceraldehydes, etc.—form dioxan-like structures in solution (Gardiner, 1966) which could react readily with oxygen to form cytotoxic peroxides. For example, the antibacterial action of hydroxyaldehydes is reduced by catalase or histidine. Further, when solutions of hydroxyaldehydes are boiled or autoclaved, their antibacterial action is considerably enhanced (Schubert, unpublished data).

Amino acids, peptides and proteins

The products formed by the irradiation of amino acids depend on the presence or absence of oxygen. Irradiation of simple, α-amino acids in oxygen-free, aqueous solution leads to both oxidative and reductive deamination so that, apart from ammonia, the major degradation products are the corresponding keto- and fatty acids, as well as hydrogen. When oxygen is present, the reductive deamination reactions do not take place because e^-_{aq} and the hydrogen atom are preferentially scavenged. Hence, the major products include ammonia and the corresponding carbonyl compound (Garrison, 1968). The higher yield of hydrogen peroxide in the oxygenated solution is also important because it can react to form adducts with the unreacted amino acids, and, in fact, many other compounds including peptides and proteins.

The aromatic amino acids, with the exception of histidine and the sulfur-containing amino acids, undergo little deamination at the α-carbon position upon irradiation whether oxygen is present or not. With the sulfur-containing amino acids, cysteine and cystine, the sulfur atom is the preferred site of reaction.

The presence or absence of oxygen is an important factor in the radiation chemistry of peptides. In oxygen, relatively high yields of ammonia and carbonyl products are found along with cleavage of the peptide "backbone". A peroxy radical is produced as an intermediate which degrades to an acrylic acid residue which in turn hydrolyses to give ammonia and a carbonyl compound. In the case of poly-γ-L-glutamic acid irradiated in neutral, oxygenated solution, the OH radical attacks the –CH bond to the side-chain carboxyl group to give the γ-peroxy radical (Garrison, 1968). The yields of ammonia and keto-compounds decrease below pH 6 because of a change in configuration from a random coil, where the peroxy radicals are free to interact both intermolecularly and intramolecularly, to a helix configuration at a pH of about 6. In the helix form, the RO_2 radicals are in a fixed position, increasing the competing reactions with water which terminate the reaction, giving less carbonyls and ammonia.

In the absence of oxygen, scarcely any cleavage of the peptide bond occurs. The major reactions are reductive deamination of the *N*-terminal amino acids giving the acyl peptide followed by recombination between the methyl, methylene, and methiol groups of the amino acids and aliphatic acids participating in the peptide bond to give a series of new peptides (Liebster & Kopoldová, 1964).

A peptide containing methionine is somewhat less radiosensitive in deoxygenated solutions than a corresponding peptide without sulfur. Further, the usual recombination and carboxylation reactions are suppressed. It is assumed that reparative processes on the sulfur atom account for these findings (Liebster & Kopoldová, 1964). In oxygenated solutions, the effects of irradiation on the sulfur-containing peptides is similar to that found in the sulfur-free peptides. Unlike other peptides, however, the reaction products found are those from methionine only whereas in sulfur-free peptides the other amino acids contribute to the reaction products.

Irradiation of proteins produces changes similar to those found in peptides—modification of side-chains, production of new groups, splitting of peptide bonds, the formation of inter- and intramolecular cross-links and breaking of hydrogen bonds. Just as in polypeptides, the steric conformation of the proteins is also important in that many reactive groups are inaccessible to free radicals or gaseous

products. Groups with labile hydrogen atoms such as –SH donate a hydrogen atom to radiation-induced radicals converting them to stable molecules. Repair of a damaged protein molecule by –SH compounds can take place. However, in the presence of oxygen, this type of repair is rare because of the reaction of the protein radical with oxygen.

Just as described earlier for peptides, both carbonyl and amide groups are formed when protein is irradiated in the presence of oxygen but in the absence of oxygen their yield is greatly reduced.

The most radiosensitive amino acid residues are generally histidine, cystine, methionine, phenylalanine, tyrosine and threonine. The principal reactions involving the OH radical are (Alexander & Lett, 1967): oxidation of the disulfide bond, substitution in the aromatic residues of tyrosine and phenylalanine, and disruption of the imidazole and indole rings of histidine and tryptophane, respectively.

The reactivity of the hydrated election with proteins includes a dependence on pH, the higher the pH the greater is the reactivity, i.e., the more loosely the hydrogen is bonded to the nitrogen, the higher is the reactivity of the group with the hydrated electron. When the pH is neutral only 3 amino acids react rapidly with e^-_{aq}, namely, cystine, cysteine and histidine (Braams, 1966).

Histidine is particularly sensitive to pH because of the ionization of the amino group in the imidazole ring which has a pK_a of 6.00, thus at neutral pH, the histidine molecule has a net positive charge. The rate constant for reactions of e^-_{aq} with the ionized form of the imidazole group is 4.3×10^9 M^{-1} sec^{-1} while the non-ionized form has a K of only 2.4×10^7 M^{-1} sec^{-1} (Braams, 1966).

Nucleic acids and constituents

Nucleoprotein is readily damaged by ionizing radiation (Scholes, 1963; Alexander & Lett, 1967; Weiss, 1964). In fact, the protein appears to protect the DNA from radiation damage. At high doses of radiation (about 0.4 Mrad) the protein precipitates. At this point, chemical attack on the bases of DNA becomes more evident. Irradiation in the presence of oxygen destroys the pyrimidine bases. A principal manifestation is a decrease in UV absorption which reflects destruction of the pyrimidine ring. While little quantitative difference exists in the extent of destruction of the various pyrimidines, thymine forms a particularly stable hydroperoxide which can be isolated and, in fact, different isomeric forms have been synthesized (Scholes, 1963). Purines are more resistant than pyrimidines to irradiation. While hydroperoxides are presumably formed upon irradiation, none have been detected because of their supposed instability. When thymine is irradiated in the absence of oxygen, the destruction in a neutral solution is about a third that observed in an oxygenated solution. The formation of thymine hydroperoxide by irradiation in oxygen is pH-dependent. The yield increases with increasing pH and reaches a maximum at a pH of about 6 and then levels off (Scholes, 1963).

The destruction of bases in nucleosides and nucleotides appears to proceed as readily as that of the simple bases. The sugar components are also damaged and the resulting compounds hydrolyse and liberate free bases. Apart from inorganic phosphate, labile phosphate esters are also formed which decompose slowly in neutral solution, eventually releasing inorganic phosphates. Irradiation of DNA in the presence of oxygen produces a hydroxy peroxide, presumably derived from the thymine component. It was possible to isolate this peroxide by precipitation of DNA with trichloroacetic acid. In the absence of oxygen, irradiation produced the aminoform-amidopyrimidine derivative which is derived from the destruction of the imidazole ring in guanine.

Radiation damage to DNA therefore encompasses the breakage of a variety of bonds—the sugar–phosphate linkage, the sugar-base, hydrogen bonds, etc. According to Alexander & Lett (1967), the chemical nature of the radiation damage observed in constituents of DNA can be extrapolated to describe the damage observed in DNA itself.

Fats and fatty acids

The formation of lipid peroxides upon ultraviolet or high-energy irradiation is well known (Mead, 1961). These peroxides are radiomimetic and inactivate enzymes, destroy sulfhydryl groups, etc. Termination of the chain reactions may take place by bimolecular interactions of the chain-carrying peroxy radicals (Hyde & Verdin, 1968).

The appearance of ketones arises in the termination step by interaction of the peroxy radicals via their secondary carbon atom to produce a molecule of α, β-unsaturated ketone. Emulsification greatly increases the rate of oxidation of irradiated methyl oleate (Hyde & Verdin, 1968).

Irradiation of solid compounds

Electron-spin resonance is used to measure free radicals formed in solid substances by radiation.

Some of these radicals persist in the solid material because of their slow diffusion but they react more rapidly when the irradiated material is dissolved.

Like the radiolysis of neutral solutions irradiated in the absence of oxygen, the irradiation of solid amino acids *in vacuo* produces ammonia and other products. Radiolysis of solid peptides produces much less free ammonia than is produced from free, solid α-amino acids. However, ammonia is a secondary product resulting from the formation and hydrolysis of labile amide-like products.

When solid proteins are irradiated in the absence of oxygen and subsequently exposed to oxygen, a peroxide radical seems to form. Actually, oxygen interferes with radiation events in proteins in many ways (Henriksen, 1967): (1) with the reactions leading to the primary radicals; (2) interaction with the primary ESR centres; (3) in secondary radical reactions; (4) reaction with secondary radicals. The rate of reaction between oxygen and a radiation-induced radical is diffusion-controlled. The diffusion of gases through crystalline forms of a polymer is much slower than through amorphous forms.

In solid serum albumin, peroxide groups introduced by irradiation in the presence of oxygen, but not *in vacuo*, are detected by the ability of the irradiated protein to induce polymerization of methacrylic acid (Alexander et al., 1956). One of the most characteristic changes induced by ionizing radiation is the increased reactivity of some of the disulfide bonds which in the native protein are inaccessible (Alexander & Hamilton, 1960).

Irradiation of solid polysaccharides leads to bond rupture but without cross-linking. While degradation appears to be enhanced in the absence of oxygen, post-irradiation effects can make large modifications. For example, if irradiation is carried out in the absence of oxygen and the polysaccharide is then stored under oxygen-free conditions, no post-irradiation changes occur. However, if the material is stored in *dry* oxygen, the viscosity falls continuously for about 10 days. In the presence of oxygen *and* water vapour, the after-effect is suppressed (Alexander & Lett, 1967). In fact, very complicated changes occur during and after the irradiation of natural biopolymers. Peroxy derivatives and formation of hydrogen peroxide can lead to degradation, depending on whether irradiation has been carried out in the presence of water vapour, as was done with dextran by Flynn, Wall & Morrow (1967).

Irradiated simple polycrystalline carbohydrates consume oxygen when dissolved in water. It appears that aqueous solutions formed by the solution of irradiated solid carbohydrates contain organic peroxides and a large variety of neutral and acidic compounds (Löfroth, 1967).

Irradiation of foods

Some of the factors which must be considered in irradiation of foods, if consistent and reproducible data are to be obtained, include the oxygen tension within a given food, the moisture content, trace element levels (especially metals), pH, chemical and biological changes as a function of post-irradiation time. The kinds of analyses ideally desired include: identification and quantification of the initial radiolytic degradation products and the secondary products produced in the irradiated foods as a function of time after irradiation; microbiological and cytogenetic toxicity, if any, of the original food and pressings; chemical identification of the toxic agent or agents and the variation with post-irradiation time. Finally, studies would be required on the efficacy of simple chemical and physical treatments to prevent the formation of harmful or flavour-destroying agents produced radiolytically, or to destroy them.

An interesting review of the chemical changes in irradiated meats has been reported (Gould, 1967, p. 121). Many of these chemical changes can be predicted from radiation chemical studies on simple systems. Some of the reported observations include radiolytic degradation of amino acids with almost total loss of methionine and cysteine and a marked loss of histidine. Irradiation of lipids and fats gives rise to oxidation products, particularly peroxides. Volatile compounds produced by the irradiation of beef include hydrogen sulfide, mercaptans, ammonia, amines, hydrogen and carbon dioxide.

Other irradiation-induced changes in foods include modification or destruction of myoglobin, vitamins, particularly E and K, naturally occurring antioxidants, and carbohydrates and organic acids, especially ascorbic and citric acids of lemons (Joint Committee on Atomic Energy, 1968, p. 123) but not of oranges.

With doses up to about 200 krad, apparently only marginal chemical changes are found in irradiated foods, though loss of tissue structure is noted at relatively low doses. In fruits, e.g., banana flesh or strawberries, as little as 50 krad and 200 krad, respectively, appeared to produce a small increase in niacin content, presumably because of increased extractability (International Symposium on Food Irradiation, 1966).

There has always been a good deal of interest in the production of peroxides in irradiated foods, particularly fats. Unfortunately, there do not exist satisfactory procedures for the quantitative analysis of peroxides as such in foods, tissues and other biologically complex media. Usually, peroxide number is used as a criterion of peroxide formation. Several measurements on the peroxide number and carbonyl compound production in irradiated fats are available and have been reviewed by Mead (1961). Apart from peroxide values for pure fats and oils, those for soybean oil, peanut oil, oleomargarine, animal fats (cooked and uncooked), lard, butter and mackerel oil are also given.

It is of interest to note that the same effects of oxygen and time are noted as would be expected in simpler systems. For example, the peroxide number of lard irradiated in the presence of oxygen is about 6 times higher than that when lard is irradiated in the presence of nitrogen, and there is a more rapid decrease of peroxide number in butter stored at 20°C compared with storage at 0°C. On the other hand, a more rapid disappearance of radiation-induced peroxides may mean a corresponding increase in secondary products.

Formation of peroxide adducts

Hydrogen and organic peroxides are formed in irradiated media. It has been found (Schubert, Watson & Baecker, 1969; Schubert, unpublished data) that hydrogen peroxide readily reacts with numerous compounds in biological media to form stable adducts. Many of these adducts are cytotoxic and mutagenic. The stability of the adducts depends on the availability of hydrogen to form a hydrogen-bonded structure between the peroxide oxygens and the hydrogen attached to the bridgehead atoms, e.g., nitrogen or oxygen. Hence, the stability depends on the pK_a of the donor atom.

Normal chemical methods are unable to detect adduct formation inasmuch as the concentrations involved, e.g., a few micrograms per millilitre of hydrogen peroxide, are too small. Further, the adducts are destroyed, albeit somewhat more slowly, by catalase. By the use of a glucose competition test (Schubert, unpublished data), it is possible to detect adduct formation. In neutral solution, stable adducts are formed between hydrogen peroxide and many amino acids, proteins, peptides, carboxylic acids and nucleic acid bases, especially thymine. Some of these adducts are even more stable in acidic media (about pH 3) than in neutral media.

Other substances such as amines, sulfur-containing amino acids and transition metals, often accelerate the destruction of hydrogen peroxide. Hence, in an irradiated food, it becomes difficult to predict the fate of hydrogen peroxide.

Organic peroxides also react with amino acids; in such cases, the reaction may result in the destruction of the organic peroxide. The reason appears to lie in the fact that it takes much less energy to rupture the O–O bond in an organic peroxide than in hydrogen peroxide (Mageli & Sheppard, 1967). A biological manifestation of the destruction of organic peroxides is shown by the fact (Schubert & Watson, 1969b) that histidine is more effective than catalase in reducing or eliminating the antibacterial action of irradiated sugars. A contributing factor may be the ability of histidine to react with carbonyl compounds as well to form stable addition compounds.

It is apparent that the formation of adducts can occur in numerous ways in irradiated foods. These adducts, which may result in either the enhancement or diminution of harmful biological effects, emphasize the fact that biological assays will remain for some time, the most direct and useful means for evaluating the safety of irradiated foods.

SELECTION OF MUTAGENICITY TESTS

An assessment of the potential mutagenic hazards of irradiated foods includes the same factors as are involved in other mutagens to which humans are exposed, namely, food additives, pesticides, ionizing radiation, drugs, air- and water-pollutants. The usual toxicity tests which measure acute, short-term, and chronic toxicity in terms of growth rate, reproductive capacity, pathological changes, haematology and the like are generally inadequate and insensitive for detecting permanent genetic damage. Many substances are classified as safe only because the testing protocol omits tests for assessing permanent genetic damage.

Since DNA is believed to have the same chemical composition in all living organisms, whether man, mouse or microbe, a chemical which damages DNA in one species can be detrimental to DNA in any other species—provided that the chemical reaches the DNA. Sometimes, the chemical itself may not be toxic, the toxicity being due rather to a derivative formed in the course of metabolism. If this is known to be the case in man, then unless a given test species is capable of metabolically transforming

such a chemical into the active form, it cannot be employed for testing unless the active form itself is administered. In other cases, a metabolic transformation or detoxification may not be manifested until after prolonged feeding, e.g., enzyme induction (Goldstein, Aranow & Kalman, 1968, chapter 3). Whether or not a substance is mutagenic can rarely become apparent in man because the statistical nature of the process may mask or hide a cause-and-effect relationship. Further, many effects may not be manifested in man for many years. Hence, we must usually rely on a variety of specialized experiments with animals having shorter life-spans. Even with relatively short-lived mammals, however, such as the mouse, it is not practicable to test for recessive mutations because of the enormous number of animals required.

The induction of chromosomal abnormalities by irradiated food or food components indicates a potential danger to man. While present procedures for screening mutagenic agents still require improvement, standardization and testing, suitable procedures are now available for such tests. While no individual test by itself suffices, a combination of tests carried out concurrently would reduce the risk that the population would be exposed to genetic hazards.

In the selection of mutagenicity tests for current use, the following requirements were considered: (1) the tests should be capable of incorporation into the usual feeding tests used for evaluating the safety of irradiated foods for human consumption; (2) the tests should be carried out *in vivo* in mammals; (3) the irradiated food or extracts prepared from them should be given by mouth; (4) the tests should be made in a reasonable time in existing biological–biochemical laboratories with relatively few animals; (5) the results of the combined tests should be capable of detecting most mutagenic agents; (6) it would be well if the tests were also prolonged so that the population would be exposed to the irradiated food during its entire lifespan. If this procedure were followed, it would mean that mutagenic tests would be made on animals removed from the normal multigeneration feeding programme at selected intervals.

Legator & Malling (1969) have pointed out that while present methods for detecting genetic damage in mammalian populations are limited, procedures are available for evaluating part of the potential danger of mutagenic agents in mammals. The many tests for mutagenicity which are available will be described in a monograph soon to be published (Hollaender, Freese & Legator, 1970). Those chosen for present use (see p. 896) are particularly relevant to man, and in addition to their practicability, are relatively simple to carry out. In terms of their sensitivity for detecting mutagenic compounds, they fall in the following order: host-mediated assay>cytogenetic>dominant lethal. Procedures for carrying out these tests are described in the Annex, primarily as an outline to inform those not acquainted with such tests. In view of the rapid developments in this field, it is expected that these tests will be modified in the future.

The limitation of the 3 tests chosen, and described below, follow the discussion given by Legator & Malling (1969).

Host-mediated assay

This test, described briefly on p. 874 and in more detail in the Annex, is quick and simple, and highly suitable for screening purposes. By comparing the mutagenic action of a compound on the microorganism directly and in the host-mediated assay, information is obtained on the rapidity with which the host can detoxify the compound and whether the host forms mutagenic breakdown products during the metabolism of the compound. The test, while flexible and applicable to a wide range of animal species and microbial indicators, is an indirect test for the detection of point mutations. Further, the ability or lack of ability of an animal to repair genetic damage cannot be determined.

Cytogenetic tests

Viable cells which show exchange figures or single-chromatid breaks are very important because such aberrations are capable of transmitting mutations to the progeny. Cells with multiple breaks are usually non-viable and hence do not constitute a genetic threat to future progeny. By including cytogenetic tests in the relatively insensitive dominant-lethal test, described below, a significant increase in sensitivity and utility can be obtained.

Dominant-lethal test

As pointed out by Generoso & Russell (unpublished data), the dominant-lethal procedure screens mainly for induced genetic change in parental germ cells that lead to chromosomal elimination and death among some of the first-generation offspring of animals treated with certain mutagenic agents. This is a useful test for detecting the mutagenicity of chemicals because it is cheap, simple and quick.

It screens mainly for chromosome breakage and will only respond to highly mutagenic chemicals. However, compounds which do give a positive response must be considered to be significant mutagenic hazards.

The early post-implantation loss is due to structural abnormalities of the chromosomes. Those abnormalities which do not kill the embryo would not be detected by this procedure. However, Legator & Malling (1969) suggest that the usefulness of the dominant lethal test can be enhanced by carrying out cytogenetic evaluations on the *normal* progeny at mid-term pregnancy when the pre-implantation and early post-implantation losses are assessed. Cytogenetic effects not severe enough to cause early post-implantation loss are probably of greater significance in terms of transmittable mutagenic damage.

General considerations

If an irradiated food gives a positive or negative test for mutagenicity in any of the 3 tests mentioned above, the question still arises whether the results are applicable to man. Obviously, it is necessary to know whether a mammal metabolizes the radiolytic products to mutagenic forms or converts mutagenic compounds to inactive forms. In such cases, *in vivo* tests can help to clarify the situation.

If a given irradiated food appears mutagenic in the test systems, the degree of mutagenicity must be evaluated. This in turn involves the question of benefit against risk. For ionizing radiation, a level of radiation which might increase the mutation rate by a factor as high as 2 has been accepted as a maximum permissible level. However, the natural unavoidable background levels of radiation to which man is exposed are known but no such baseline data are available for irradiated foods. However, categories of risk for the consumption of irradiated foods can be considered as follows.

Category 1. The irradiated food which would be consumed by all segments of the population regardless of age, state of health, pregnancy, etc., gives a positive test for mutagenicity.

Category 2. As in category 1, but only weakly mutagenic.

Category 3. The irradiated food is available only to a small restricted group of the population, based, for example, on age or special nutritional needs.

It appears prudent to recommend that no food in category 1 should be released for consumption, at least until further tests to evaluate the risk have been completed. Items which fall into category 3 can generally be consumed, assuming restricted or controlled distribution. Items in category 2 fall in an ill-defined area in which the benefit versus risks must be carefully evaluated for each case.

FUTURE RESEARCH

Mutagenic testing will eventually become incorporated into protocols, not only for irradiated foods but also for foods processed by other techniques, as well as those affected by other environmental agents. Considerable research is needed to improve techniques, establish bases for extrapolating the results from animal testing to man, etc. Some of the problems for which further research and development are needed include the following.

(1) The standardization of *in vivo* and *in vitro* mutagenic screening tests.

(2) Correlation between *in vivo* and *in vitro* systems using mammals and mammalian cells.

(3) Suitability of different mammals for *in vivo* detection of chromosomal aberrations, e.g., mammals such as the rat-kangaroo with low chromosome numbers, in order to facilitate analysis of aneuploidy.

(4) Metabolic transformations undergone in mammals after the feeding of suspected chemical mutagens, and the identification of mutagenically active and inactive forms.

(5) The time at which a mutagenic response may appear or disappear following prolonged or chronic administration of suspected mutagens.

(6) Development of new or improved indicators of mutagenesis in the host-mediated assay test.

(7) Mutagenic testing of unirradiated foods and food components which have been treated or processed by canning, smoking, etc.

(8) Search for synergistic action or antagonistic action, e.g., irradiated foods plus caffeine, irradiated foods and alcohol, or irradiation plus heat.

(9) Testing and search for chemicals capable of counteracting the action of mutagens.

(10) Intracellular localization or distribution of a chemical mutagen or its metabolically active products.

(11) Development of reliable and adequately sensitive chemical methods for the detection and quan-

tification of compounds produced in the irradiation of foods, e.g., organic peroxides.

(12) Screening of synthetic compounds of the kinds expected to be found in irradiated foods such as the adducts of hydrogen peroxide with amino acids, peptides, proteins, nucleic acid bases, carboxylic acids, etc. These adducts need not be prepared as pure compounds; the adduct-forming compound is simply added to a buffered solution of hydrogen peroxide at a 5-fold greater concentration on a molar basis than the hydrogen peroxide.

(13) Effect of chemical mutagen concentration relative to cell concentration on the mutagenic response.

(14) Mechanisms, both chemical and physical, by which chromosomal aberrations are produced.

Annex

OUTLINE OF METHODS FOR THE DETECTION OF MUTAGENICITY OF IRRADIATED FOODS *IN VIVO*

Laboratory procedures for 3 tests are presented here to illustrate the kind of effort required. In practice, various laboratories and investigators may modify these tests, but it is hoped that some standardization may be established in the future. Regardless of the test performed, certain control experiments must be included. First, controls receiving the identical non-irradiated food must be run in parallel. Second, a positive control using a mutagenic compound, e.g., an ethyleneimine alkylating agent, should be included in every run. This is to support negative results, i.e., so that the lack of mutagenicity cannot be due to inadequacies in the test system or to the techniques employed.

Just as in the feeding tests, at least 2 species must be employed for each test. In the case of the host-mediated assay, it is suggested that 2 species of host should be employed, but also at least 2 different micro-organisms. The irradiated foods or preparations made from them should be administered orally *via* a stomach tube. If the food is a solid, it should be mixed with an equal weight of distilled water and made into a slurry, suspension or homogenate. A "moist" food can be homogenized directly. Mice can be given 0.5 ml–1 ml of the food 3 times daily. Larger animals can be given larger volumes, e.g., 4 ml on each occasion to rats.

While the procedures given are for short-term testing, it is recommended that the protocol for feeding tests should include additional animals. Twice a year, a selected number of animals and their offspring should be tested for mutagenicity by the 3 tests described here, or by other tests that may become available.

Host-mediated assay

In this method (Gabridge & Legator, 1969), an indicator micro-organism is incorporated within a mammalian (murine) host. Direct administration of the compound to the host allows the animal to activate or detoxify the potential mutagen before it encounters the micro-organisms in the peritoneum. After a 2-hour incubation period, the micro-organisms are removed aseptically from the peritoneum and placed on minimal and complete media to determine the ratio of mutants to wild type. The resulting mutant frequency is compared with that occurring spontaneously and thus serves as an indicator of relative mutagenicity.

A nonsense mutant [1] of *S. typhimurium* G46 has been used (Whitfield, Martin & Ames, 1966). It is a stable, well-characterized strain. Another usable micro-organism is *E. coli* 532, a streptomycin-dependent strain. A host-mediated assay method using *Neurospora* is described below.

Procedure. Mice, rats and guinea-pigs can be used. The procedure described here is for the mouse using *S. typhimurium.* When larger species are used, the only modification is to increase the titre (not the volume) of the micro-organism suspension in proportion to body weight.

Tryptone broth is inoculated from an agar-slant culture of *S. typhimurium* and incubated in a reciprocating shaker for 2 hours at 37°C. This culture is diluted in the ratio 1 : 4 with saline (the final OD_{660}

[1] A nonsense mutation refers to a base triplet (codon) which changes to a codon that does not correspond to any amino acid.

is about 0.1) and 2.0 ml of the resulting suspension is used for intraperitoneal inoculation into the mouse.

A technique using *Neurospora* is being tested at the Oak Ridge National Laboratory, USA. The *Neurospora* conidia are suspended in Hanks' solution and injected into the peritoneum of mice or rats and into the testis of rats. The percentage survival after 24 hours under these conditions is close to 100 and 50%–70% of the conidia can be recovered (Malling, personal communication).

Male mice weighing 25 g–30 g are treated in groups of 4. A 1-ml preparation of the irradiated food is administered by stomach tube to each animal and is given twice at 2-hourly intervals. Two hours after the injection, each mouse receives 1.0 ml of saline intraperitoneally and as much fluid as possible is aseptically removed from the peritoneum and pooled before titrating.

Another group of mice is fed 3 times daily for 6 days. On the seventh day, the mice are fed twice and then the test is run and described.

Ten-fold serial dilutions (10^{-1}–10^{-7}) of the peritoneal fluid are made in saline. The 4 highest dilutions are plated on minimal agar with a histidine overlay to give the total *Salmonella* cell count, and the 3 lowest dilutions (including 10^{0}) are plated on minimal agar without histidine for *Salmonella* mutant growth.

Plating techniques. All plates (15 mm by 100 mm plastic Petri dishes) contain a base layer of 20 ml of minimal agar.[1] A standard pour-plate technique is used: 0.1 ml of the proper dilution and 2.0 ml of 0.6% molten agar are added to a sterile tube, mixed, and poured over the surface of a base plate. The histidine overlay contains 0.1 ml of 0.1M L-histidine per ml of overlay agar. After the contents solidify, the plates are incubated at 37°C for 48 hours, and the ratio of mutants to total cells is determined.

The incubation technique (de Serres & Osterbind, 1932) using *Neurospora* is performed as follows: The *Neurospora* conidia inoculated into 12-litre flat-bottomed flasks with Westergaard's minimal medium supplemented with adenine. Specific locus mutations in the adenine-3 region of the *Neurospora* chromosome are detected as purple colonies among the unmutated white colonies. The mutants can arise as a result of a point mutation or a chromosome deletion. This is the only existing technique with which point mutations and chromosome deletions can be detected simultaneously in the same region of the chromosome.

[1] Spizizen's medium is prepared as follows (4X salt solution): ammonium sulfate, 8.0 g; dibasic potassium phosphate, 56.0 g; monobasic potassium phosphate, 24.0 g; sodium citrate, 4.0 g; magnesium sulfate, 0.8 g; and water is added to 1000 ml. Agar (15 g) is added to 650 ml of water and the mixture is autoclaved. To prepare the final mixture, 100 ml of sterile 5% glucose and 250 ml of sterile 4X salt solution are added to the agar solution.

Dominant lethal test

Procedure 1 described here is based on that reported by Generoso & Russell (1969) while procedure 2 is the one devised by Epstein et al. (unpublished data) and modified from a previously published procedure (Epstein & Shafner, 1968) In any case, the animals are given the irradiated food by intubation 3 times daily for 4 days. Animals fed chronically in the long-term feeding experiments are also used at appropriate times. Appropriate numbers of concurrent controls should be used.

Procedure 1. Immediately after treatment each male is paired with a suitable female. Every morning, females are examined for presence of vaginal plugs (indication of mating) and each female which has copulated is removed and replaced by a virgin female. This routine is carried on for a period of 7 weeks. All mated females are killed between 13 and 17 days after observation of the vaginal plug and examined for the number of living embryos and dead implantations.

" The mutagenicity of the test compound is decided by the combination of the following criteria: (1) reduction in the average number of living embryos, (2) increase in the frequency of dead implantations, (3) reduction in the average number of implantations, and (4) reduction in the frequency of fertile matings. Generally, the first and second and in some cases also the third criteria are expressed together. The fourth criterion is expressed only when dominant-lethal induction approaches 100%. Infertility of treated males may be due to dominant lethality or to physiologic reasons like the inability of treated males to mate during the post-treatment sick phase. When infertility of treated males is due to dominant lethality, the first three criteria are usually expressed among the fertile matings. Data on the frequency of matings (as indicated by vaginal plugs) will also be helpful in this case."

" It should be noted that the sensitivity of this procedure depends very heavily on the reproductive nature of the females. It is, therefore, important to use a strain of females possessing the following qualities at the age when used in the test: (1) large litter size, (2) low frequency of dead implantations, (3) high proportion of vaginal plugs which represent matings during the receptive stage of the estrous cycle, and (4) uniformity among females " (Generoso & Russell, 1969).

Procedure 2. This procedure (Epstein, private communication) is a rapid one and has been used for the screening of numerous compounds.

" Following treatment, males are individually caged with untreated virgin females for seven days; the females are then replaced with fresh females and this mating pattern is maintained for eight weeks. All females are autopsied at 13 days from the mid-week of their caging and presumptive mating. Females are scored for pregnancy, total number implants *per* pregnant female, and number of early deaths *per* pregnant female."

" Numbers of early fetal deaths *per* pregnant female constitute the most direct and precisely quantitative parameter of mutagenicity. However, reduction in the total number of implants, including living fetuses and early fetal deaths, as compared with controls, provides a measure of pre-implantation loss which in general is a presumptive index of mutagenic effects."

As mentioned earlier (p. 895), the inclusion of cytogenetic testing of the normal progeny at mid-term of pregnancy can enhance the usefulness and sensitivity of the dominant lethal test.

Rats are also used as test animals. According to Legator (personal communication), rats are very convenient to handle and the resorption rate is easier to read. The autopsies are made 13 days from the mid-week of mating, just as in mice. The rats are mated with 2 females per week, so that more males are needed to produce the same number of pregnancies.

Cytogenetic techniques

Male animals, 11–12 weeks old, are used so that chromosome analyses can be carried out on both the bone marrow cells and the germ cells of the same animal. The animals, 5 to a group, are given, by intubation, homogenates of the irradiated food 3 times daily beginning on a Monday morning. Twenty hours later, samples are taken for chromosome analysis. Daily feeding of the animals continues. On Friday morning samples are again taken for chromosome analysis. The animals are fed on the Friday, but not on Saturday or Sunday. The final samples for chromosome analysis are taken on Monday.

There are many techniques for studying chromosomes (Moorhead & Nowell, 1964). The object of all the techniques is to obtain numerous intact, well-fixed, dividing cells containing well-separated chromosomes that can be analysed. Depending on the purposes of the study there are many tissues of the body that can be used for the preparation of chromosomes. For example, in long-term research observations, skin fibroblasts provide these data, while for convenient routine clinical diagnosis, peripheral leucocytes or bone marrow cells are satisfactory. Different cytogenetic techniques must be used for different tissues.

In order to obtain numerous dividing cells in short-term leucocyte cultures, mitosis is stimulated by phytohaemagglutinin (PHA). To produce many chromosomes suitable for analysis at the mitotic metaphase, colchicine or colcemide is used to disrupt the spindle mechanism. A more pronounced dispersion of the chromosomes in the mitotic cell can be achieved by pretreatment with a hypotonic solution before fixation. Distilled water, tap-water, or hypotonic solutions of sodium citrate are all very effective. The choice depends on the type of tissue and individual preference. For bone marrow and peripheral blood culture, sodium citrate is most useful. Fixation and staining take place after the hypotonic pretreatment. Acetic-acid orcein, Feulgen and Giemsa reagents are the most popular stains, Giemsa being used mainly for blood cells. After fixation, the spreading of chromosomes in one plane on a slide can be achieved by 2 methods; external pressure (squash) or air-drying. The air-drying method is preferable for peripheral blood leucocytes, bone marrow and fragile cells. Analysis of chromosomes should include counting and a morphological examination for structural abnormalities.

To obtain peripheral blood for cytogenetic studies in the laboratory mouse from the tail or orbit, or by heart puncture, requires expert technique and patience, and the culture of the small quantity available is not always successful. Even if the culture is satisfactory, it requires a 5–6-day incubation period. Chromosome analysis using bone marrow cells has the great advantage of being both quicker and technically simpler than it is when cultured peripheral blood is used. Generally, however, the marrow has only been obtained after sacrificing the animal, removing the long bones and opening them. This precludes any serial chromosomal observations.

Procedure for bone marrow. In the technique developed by Pan & Wald (1963), bone marrow from the femur and humerus of the mouse is obtained by aspiration without sacrificing the animal; serial chromosome observations can therefore be made.

(1) Pin a mouse anaesthetized with ether on a board with its abdomen down and hind legs stretched out.

(2) Make a small longitudinal incision in the skin on the dorsolateral surface of the thigh, long enough

to expose the muscles of the lower half of the thigh and a small portion of the knee joint.

(3) Separate the superficial fascia and the muscles along their fibres by blunt dissection to expose the lower third of the femur. Haemorrhage at this stage is almost non-existent because most of the large vessels are located at the anterior and posterior medial surfaces.

(4) Drill a small hole in the exposed shaft into the marrow cavity with a sharp 22-gauge needle, using a steady controlled force. A strong or sudden force may fracture the bone.

(5) Wash and aspirate the marrow from the hole by alternately aspirating and ejecting through a blunt 25-gauge needle attached to a tuberculin syringe containing a small amount of colcemide in normal saline in a concentration of 1 μg/ml at 37°C.

(6) Repeat the washing and aspiration until the bone appears white. Collect the cell suspension in a Kahn test-tube and fill the test-tube with additional colcemide solution.

(7) Incubate the tube at 37°C for 2 hours and centrifuge it at 3400 rev/min for 90 seconds. Then carry out cytogenetic preparation as desired.

Another procedure is to aspirate marrow from the humerus. The technique is the same as for the femur except that the skin incision is made at the dorsolateral surface of the foreleg.

Cytogenetic preparation. The chromosomes are prepared for analysis as follows:

(1) Add colcemide or colchicine with a final concentration of 1 μg/ml into the tubes at the end of the incubation period.

(2) Incubate the tube at room temperature for another 3–4 hours and then centrifuge to collect the sediment.

(3) Resuspend the sediment in 0.25% sodium citrate solution for ½ hour.

(4) Centrifuge the tube at 3400 rev/min for 90 seconds and fix the sediment immediately with acetic-acid–alcohol containing 3 parts of absolute ethanol and 1 part of glacial acetic acid.

(5) Change the fixative 3 or 4 times by repeated centrifugation and resuspension.

(6) Drop and disperse a small amount of the fixed suspension on to a clean slide and let it dry in the air for a few minutes.

(7) Stain for 7–10 minutes with Giemsa stain.

(8) Dry and cover the slides with cover-slip. The slides are then ready for microscopic examination.

Discussion. The amount of marrow obtained from one femur of a mouse usually is sufficient to prepare 4–6 slides which contain a sufficient number of cells for chromosomal analysis and photography. The amount obtained from the humerus is usually less than from the femur. However, if both humeri are used, they usually yield a sufficient number of cells suitable for analysis.

The advantage of this technique, besides simplicity and speed, is that there is no need to sacrifice the animals. Therefore, marrow from the same animal can be sampled at 3 or more intervals, once from both humeri and once from each individual femur.

The time required for the regeneration of the marrow in an aspirated long-bone has not been determined definitely. On one occasion in a normal mouse, a usable amount of marrow was found 3 weeks after the first aspiration and in another mouse at 4 weeks. No apparent anaemia has been noted, although no detailed haematological follow-up examinations have been attempted.

It is not necessary to observe a strictly sterile technique for the aspiration. The wound clip on the skin incision can be removed on the third or fourth post-operative day. The hole in the bone shaft heals completely in 3–4 days. When the bone is accidentally fractured, it unites spontaneously in 4 days and the site of the fracture is surrounded by new bone.

Testicular aspiration procedure. The procedure described here, by not requiring removal of the testes, permits serial chromosome analyses on the germ cells to be made just as in the case of the bone marrow cells. The method, developed by Pan (unpublished data), is based on that described by Evans, Breckon & Ford (1964).

Modified procedure. The procedure described here (Pan, unpublished data) has been developed for mice. For use with different species, modification in the amounts of colcemide administered must be made. For full details regarding fixatives and other details, see the article by Evans, Breckon & Ford (*op. cit.*).

(1) Inoculate the mouse with 0.5 ml of 0.025% colcemide 2 hours before sampling.

(2) Aspirate 1 testis using a 22-gauge needle with a 2-ml syringe.

(3) Centrifuge the cell suspension at 500 rev/min for 90 seconds. Discard the supernate and resuspend in 1% sodium citrate.

(4) Treat for 12 minutes with a hypotonic solution at room temperature.

(5) Add fixative diluted 1 : 3 for 30 minutes and rinse several times with fresh fixative.

(6) Blow out cell suspensions on to a standard microscope slide.

(7) Hydrolyse.

(8) Stain with Wright's, Geimsa or Feulgen reagents.

Chromosome analysis. The slides are scanned, using the low-power objective of the microscope, and the mitotic figures encountered, generally in metaphase, are studied in detail using an oil-immersion lens. Counting of chromosomes and the examination of any aberrations may be done by direct observation, micro-projection or by photomicrography. Polaroid photomicrographs and 35-mm enlargements are used and analysed for abnormalities. A minimum of 50 metaphase plates selected at random are analysed.

In cytogenetic analysis, 4 levels of significance or importance can be determined (Legator & Jacobson, 1969).

(1) The *greatest significance* is attributed to persistent clonal aneusomy, second-generation translocation figures (exchanges), structural markers (dicentrics, rings) and endoreduplications.

(2) *Moderate significance* is awarded to non-clonal hyperdiploidy, persistent polyploidy, fragmentation and chromosomal (isochromatid) breaks at non-secondary construction sites; also to inconsistent karyotypic anomalies suggesting deletion or translocation.

(3) *Mild significance* is attributed to chromatid breaks, telomere blebs, chromatid fragments and to non-clonal hypodiploidy.

(4) *No significance* has been attributed to chromosome gaps and sporadic homologue asymmetry, i.e., morphological differences between the two members of a chromosome pair.

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RÉSUMÉ

POUVOIR MUTAGÈNE ET CYTOTOXICITÉ DES ALIMENTS ET DES COMPOSANTS ALIMENTAIRES IRRADIÉS

L'auteur procède à une revue détaillée et critique *a*) des effets mutagènes et cytotoxiques des aliments et composants alimentaires irradiés, *b*) des principes et des applications des tests destinés à étudier le pouvoir mutagène *in vivo* chez les mammifères, et *c*) des réactions chimiques induites qui se produisent dans les milieux irradiés, l'accent étant mis notamment sur la nature des facteurs mutagènes et cytotoxiques présumés. Ce travail a pour but de faciliter l'évaluation de l'innocuité des aliments irradiés et de définir les domaines où de plus amples recherches sont nécessaires.

Le problème d'une éventuelle action mutagène ne se pose pas uniquement pour les aliments irradiés. Toute une série de facteurs de milieu représentent à cet égard une menace identique pour l'homme: additifs alimentaires, pesticides, médicaments, polluants de l'atmosphère et de l'eau, aliments traités par d'autres procédés, comme la chaleur par exemple. De nouvelles évaluations de l'innocuité de toutes les substances quant à leur capacité d'induire des mutations sont devenues indispensables depuis qu'on sait que les épreuves classiques de toxicité sont impuissantes à déceler des risques insidieux comme les effets mutagènes et tératogènes.

Un grand nombre de cellules — de mammifères, de

végétaux ou bactériennes — sont détruites ou lésées quand on les place dans un milieu préalablement exposé aux rayonnements ionisants ou aux rayons ultraviolets. Cette aptitude des rayonnements à agir indirectement sur les molécules et les organismes par irradiation préalable du milieu est appelée « effet de milieu » ou « effet indirect » ou encore « effet retardé ». Cet effet peut se manifester de diverses façons: transformation de macromolécules, lésions génétiques (mutations et altérations chromosomiques), inhibition de la croissance bactérienne et cellulaire, modification d'un certain nombre de processus biochimiques et physiologiques.

La plupart des recherches consacrées à l'étude des effets de milieu à l'aide d'aliments et de composants alimentaires ont été faites *in vitro* et ont permis d'enregistrer de nombreuses réponses mutagènes et cytotoxiques. Cependant, la mise en évidence *in vitro* de substances nocives, comme conséquence de l'irradiation, n'implique pas nécessairement que ces mêmes substances auront aussi une action néfaste *in vivo* car il faut tenir compte d'un certain nombre d'autres facteurs et, entre autres, des processus métaboliques. Même si les rayonnements donnent naissance à un agent mutagène présumé, celui-ci peut ne pas atteindre les organes ou les cellules « cibles » à des concentrations suffisamment élevées pour entraîner des dommages génétiques. On n'a procédé qu'à un nombre relativement limité d'expériences *in vivo* sur des mammifères portant sur les effets mutagènes, comme les aberrations chromosomiques ou l'apparition de gènes létaux dominants.

Parmi les nombreux procédés d'étude *in vivo* des agents mutagènes, trois offrent actuellement un intérêt particulier, bien que sujets encore à des modifications ou des améliorations. Ce sont, par ordre d'efficacité: 1) l'épreuve par hôte interposé, consistant à implanter dans la cavité péritonéale d'un mammifère nourri à l'aide d'aliments irradiés des bactéries, des *Neurospora crassa* ou d'autres organismes, qui sont ultérieurement prélevés et examinés pour dépister d'éventuelles mutations; 2) l'analyse cytogénétique des chromosomes de cellules du testicule et de la moelle osseuse après administration d'aliments irradiés; 3) l'étude des mutations létales dominantes, grâce à des appariements d'animaux mâles recevant des aliments irradiés et de femelles vierges non traitées, avec dissection de ces dernières au milieu de la période de gestation. Cette dernière épreuve, relativement peu sensible, peut être complétée par l'étude cytogénétique similaire de la progéniture normale. Les aspects techniques de ces différents tests sont décrits.

Il est prouvé que dans certains systèmes les effets mutagènes ou cytotoxiques observés sont dus à la présence de peroxydes organiques produits par l'action du peroxyde d'hydrogène sur des composés carbonylés. On a, dans certains cas, isolé de tels peroxydes, par exemple après irradiation d'une solution oxygénée d'histidine. On consacre actuellement de nombreuses recherches à l'étude de l'activité *in vivo* des mutagènes présumés. En général, il apparaît que beaucoup des systèmes irradiés qui lèsent les chromosomes *in vitro* n'ont aucune action de ce genre lorsqu'ils sont expérimentés *in vivo* par l'intermédiaire des aliments.

D'après les données connues, il semble improbable que les faibles doses de rayonnements (10-75 krad) utilisées pour stériliser certains aliments ou empêcher la germination aient une action mutagène lorsque les produits traités sont introduits dans l'alimentation. De nombreuses recherches sont encore nécessaires afin d'améliorer les techniques et de définir les modalités d'extrapolation des résultats à l'homme. Certains aspects du problème méritent d'être spécialement étudiés, entre autres: *a*) la normalisation des épreuves de dépistage des agents mutagènes *in vitro* et *in vivo*; *b*) la recherche des corrélations entre les systèmes utilisant des mammifères ou des cellules de mammifères *in vitro* et *in vivo*; *c*) la sélection des mammifères qui se prêtent le mieux à l'étude *in vivo* des aberrations chromosomiques; *d*) la durée du délai d'apparition ou de disparition d'un effet mutagène après administration prolongée ou continue de mutagènes potentiels; *e*) l'activité mutagène éventuelle d'aliments non irradiés soumis à divers autres traitements; *f*) les transformations métaboliques subies par les mutagènes chimiques présumés après leur administration aux mammifères et l'identification de leurs formes actives et inactives.

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